Enhanced pro-apoptotic and anti-adipogenic effects of genistein plus guggulsterone in 3T3-L1 adipocytes

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Abstract. Genistein (G), an isoflavone, and guggulsterone (GS), the active substance in guggulipid, have been reported to possess therapeutic effects for obesity. In the present study, we investigated the effects of combinations of G plus GS on apoptosis and adipogenesis in 3T3-L1 cells. In mature adipocytes, G and GS individually caused apoptosis, but combination of G plus GS significantly increased apoptosis, more than either compound alone. Furthermore, G plus GS caused a greater increase in procaspase-3 cleavage, Bax expression, cytochrome c release, and proteolytic cleavage of PARP than either compound alone. In maturing preadipocytes G and GS each suppressed lipid accumulation, but the combination potentiated the inhibition of lipid accumulation. These results suggest that combination of genistein and guggulsterone may exert anti-obesity effects by inhibiting adipogenesis and inducing apoptosis in adipocytes.

Keywords: Genistein, guggul, 3T3-L1 adipocytes, apoptosis, cytochrome c, mitochondria, adipogenesis

1. Introduction

Obesity is a risk factor for severe diseases such as diabetes, atherosclerosis, coronary heart disease, and certain cancers [5]. In response to excess energy intake, adipose tissue increases in mass by hyperplasia, due to the proliferation and differentiation of preadipocytes, and by hypertrophy, due to increased storage of triglycerides [10]. When energy intake is less than energy output, adipose tissue mass decreases as a result of mobilization of triglycerides, but adipocyte number generally does not decrease with fat mobilization alone. However, under some conditions, adipose tissue can also be decreased by apoptosis of preadipocytes and adipocytes [29]. However, little is known about the mechanisms involved in the regulation of fat cell number.

Some individual natural compounds have been shown to inhibit adipocyte differentiation [15,18] and induce adipocyte apoptosis [7]. We have investigated several combinations of natural compounds that have enhanced effects on induction of apoptosis and inhibition of adipogenesis compared to the effects
Genistein, a soy isoflavone, has been shown to decrease food intake and body weight [15,21] and to increase apoptosis of adipose tissue [15] in ovariectomized female mice. Genistein has also been shown to inhibit cell proliferation and increase lipolysis in adipocytes [14]. In addition to its estrogenic effects, genistein has been shown to induce apoptosis in many types of cancer cells [30]. More recent studies have shown that genistein inhibited adipocyte differentiation via activating AMP-activated protein kinase [12], and it inhibited adipogenesis by decreasing PPARγ and C/EBPα [9].

Guggulsterone (GS), the active substance in guggulipid, has been of interest because of its effects on lipid metabolism, as guggulipid has been found to reduce triglyceride levels as well as cholesterol [32]. In one study oral administration of gum guggul alone was shown to decrease body weight in both animals and humans [25]. More recent studies have confirmed the effect on cholesterol levels and have shown that this activity is at least partly due to the antagonism of nuclear farnesoid X receptors (FXR) by guggulsterone [33,34]. Interestingly, FXR appears to play a role in the regulation of adiposity. FXR knockout mice were shown to have decreased adipose tissue mass and embryonic fibroblasts from these mice had impaired adipogenesis [4]. GS has also been shown to inhibit cell proliferation and induce apoptosis in many types of cancer cells [26].

Based on these findings, the current study was carried out to determine whether the combination of G and GS has enhanced activity in inducing apoptosis of mature adipocytes and in inhibiting adipogenesis in maturing preadipocytes. In this report, we show that the combination of genistein and guggulsterone caused an enhanced apoptosis and inhibition of adipogenesis compared to either compound alone. We also further elucidate some of the biochemical mechanisms involved in apoptosis induced by the combination of genistein and guggulsterone in mature adipocytes.

2. Materials and methods

2.1. Cell culture

3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured as described elsewhere [11]. Briefly, cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine calf serum (BCS) until confluent. Two days after confluence (D0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 167 nM insulin, 0.5 µM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone for two days (D2). Cells were then maintained in 10% FBS/DMEM medium with 167 nM insulin for another two days (D4), followed by culturing with 10% FBS/DMEM medium for an additional 4 days (D8), at which time more than 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% CO2 atmosphere.

2.2. Reagents and antibodies

PBS and DMEM medium were purchased from GIBCO (BRL Life Technologies, Grand Island, NY). Genistein (99% pure) was purchased from Indofine Chemical Company (Hillsborough, NJ). Cis-guggulsterone (99.0%) was purchased from Steraloids, Inc. (Newport, RI). ApoStrand™ ELISA Apoptosis Detection Kit was purchased from BIOMOL (Plymouth Meeting, PA). The cell viability assay kit was purchased from Promega (Madison, WI). AdipoRed assay reagent was purchased from Cambrex, Inc (Walkersville, MD). Antibodies specific for polyclonal PARP, Bax, caspase-3, β-actin, and cytochrome c were from Santa Cruz Biotechnology (Santa Cruz, CA).
2.3. Cell viability and apoptosis assays

Cells were seeded in 96 well plates at 5000 cells/well and grown to maturation as described above. Mature adipocytes were incubated with either carrier (0.2% DMSO) or test compounds. Cell viability assay was performed as per the manufacturer’s instructions. 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. For the assessment of apoptosis, the ApoStrand™ ELISA Apoptosis Detection Kit 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 [6]. Prior to ssDNA ELISA, adipocytes were incubated with either DMSO or test reagents. Thereafter, treatment medium was removed and the cells were fixed for 30 min and assayed according to the manufacturer’s instructions.

2.4. Quantification of lipid content and oil Red O staining

Postconfluent preadipocytes were grown in 96 well plates and incubated with either carrier (0.2% DMSO) or test compounds along with the induction medium for days 0–6 of adipogenesis. Lipid content was quantified on day 6 using commercially available AdipoRed™ Assay Reagent according to the manufacturer’s instructions. Oil Red O staining was performed as described by Suryawan and Hu [31]. Three images for each dish were captured using ImagePro software (MediaCybernetics, Silver spring, MD).

2.5. Western blot analysis

Whole cell extracts and cytosolic fractions were prepared as described elsewhere [35]. The protein concentration was determined by the method of Bradford with bovine serum albumin as the standard. Western blot analysis was performed using the commercial NUPAGE system (Novex/Invitrogen, Carlsbad, CA). Samples were heated to 70°C for 10 min, separated by 12% acrylamide gels and analyzed by immunoblotting.

2.6. Quantitative analysis of Western blot data

Measurement of signal intensity on PVDF membranes after Western blotting with various antibodies was performed using a FluorChem™ densitometer with the AlphaEaseFC™ image processing and analysis software (Alpha Innotech Corporation). For statistical analysis, all data were expressed as integrated density values (IDV), which were calculated as the density values of the specific protein bands/β-actin density values and expressed as percentage of the control. All figures showing quantitative analysis include data from at least three independent experiments.

2.7. Statistical analysis

ANOVA (GLM procedure, Statistica, version 6.1; StatSoft, Inc.) was used to determine significance of treatment effects and interactions. Fisher’s post hoc least significant difference test was used to determine significance of differences among means. In some cases in order to estimate differences between the combined treatments and a hypothetical additive treatment response, a sum of the individual treatment effects for each replicate was calculated and these numbers were included in the ANOVA. Statistically significant differences are defined at the 95% confidence interval. Data shown are means ± SEM.
3. Results

3.1. Genistein and guggulsterone reduce cell viability and induce apoptosis

Adipocytes were treated with carrier or genistein (50, 100 µM) and guggulsterone (25, 50 µM) as individual compounds and in combinations for 24 and 48 h. G and GS individually at the concentrations tested had no effect on decreasing cell viability (Fig. 1A). The combinations of G and GS (G+GS) significantly decreased viability dose-and time-dependently. G100+GS50 decreased cell viability by 25.8 ± 1.4% (P < 0.001) and 57.0 ± 2.7% (P < 0.01) after 24 and 48 h of treatment, respectively. We next investigated whether the reduction in cell number by G and GS was due to apoptosis. G100 and GS50 increased apoptosis by 68.9 ± 16.5% (P < 0.01) and 110.0 ± 34.7% (P < 0.001), respectively, after 48 h of treatment, while individual compounds at lower concentrations had no significant effect (Fig. 1B). G100+GS50 further increased apoptosis by 565.8 ± 17.1% (P < 0.001) whereas the calculated additive response (G100GS50) would have been an increase of only 178.9 ± 34.7%, P < 0.001 (Table 1). The enhanced effect of G100+GS50 on apoptosis was further confirmed using fluorescence microscopy of cells treated with Hoechst stain (data not shown), which stains the nucleus of apoptotic cells with a
Percent change in viability and increase in apoptosis of mature adipocytes after 48 h treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Change in Viability Mean ± SEM</th>
<th>% Increase in Apoptosis Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 ± 1.2^c</td>
<td>0.0 ± 7.3^c</td>
</tr>
<tr>
<td>G50</td>
<td>4.0 ± 2.4^a</td>
<td>3.8 ± 6.5^c</td>
</tr>
<tr>
<td>G100</td>
<td>4.2 ± 1.0^a</td>
<td>68.9 ± 16.5^a</td>
</tr>
<tr>
<td>GS50</td>
<td>14.1 ± 1.4^w</td>
<td>110.0 ± 34.7^ab</td>
</tr>
<tr>
<td>G50GS50 (calculated)*</td>
<td>18.1 ± 1.4^w</td>
<td>113.8 ± 34.7^ab</td>
</tr>
<tr>
<td>G100GS50 (calculated)*</td>
<td>18.3 ± 1.4^w</td>
<td>178.9 ± 34.7^b</td>
</tr>
<tr>
<td>G50+GS50 (combined)</td>
<td>−32.0 ± 2.1^y</td>
<td>318.2 ± 28.7^d</td>
</tr>
<tr>
<td>G100+GS50 (combined)</td>
<td>−57.0 ± 2.7^y</td>
<td>565.8 ± 17.1^e</td>
</tr>
</tbody>
</table>

^*Calculated additive responses.

wxyz: means without common letters are different, \( P < 0.01 \).

abcde: means without common letters are different, \( P < 0.05 \).

higher-intensity than that of normal cells [22]. Since G100+GS50 inhibited cell viability by 50% and induced apoptosis, these concentrations of genistein and guggulsterone were selected for subsequent apoptosis experiments.

### 3.2. Effect of genistein and guggulsterone on caspase-3 and PARP cleavage

To identify whether the effector caspase-3 is involved in G+GS-mediated apoptosis induction, we analyzed the activation of caspase-3 by Western blotting (Fig. 2A). G100 and GS50 individually had no effect on caspase-3 activation. G100+GS50 decreased pro-caspase-3 (32 kDa) level by 30.0 ± 3.2% \( (P = 0.14) \) after 24 h of treatment. The 17 kDa cleavage product, which is the active caspase-3, was significantly increased by 194.7 ± 26.6% \( (P < 0.001) \). G100+GS50 also increased the proteolytic cleavage of PARP (116 kDa) by 49.8 ± 6.1% \( (P < 0.01) \) after 48 h, resulting in increased accumulation of the 85 kDa cleavage product by 148.8 ± 20.7% \( (P < 0.001) \), while G and GS individually had no effect (Fig. 2B).

### 3.3. Involvement of the mitochondrial pathway

To further investigate whether G and GS affect the intrinsic apoptosis induction pathway, we evaluated the expression of proapoptotic Bax protein by Western blotting. Treatment with GS50 alone led to a small, but not significant, increase in Bax and G100 alone had no effect after 48 h (Fig. 3A). G100+GS50 significantly increased Bax expression by 174.3 ± 39.3% \( (P < 0.001) \). Bax has been shown to induce cytochrome c release \textit{in vitro} [13]. Since this release has been implicated in a variety of apoptotic phenomena, we also examined whether the enhanced increase in apoptosis by G+GS involved cytochrome c. G100 and GS50 individually had no effect (Fig. 3B), while G100+GS50 significantly increased cytochrome c level by 376.0 ± 21.4% \( (P < 0.001) \).

### 3.4. Genistein and guggulsterone inhibit lipid accumulation

A preliminary screening experiment revealed that maturing preadipocytes are much more sensitive than mature adipocytes, and hence we used low concentrations of genistein (25 and 50 µM) and guggulsterone (6.25 and 12.5 µM) for adipogenesis experiments. During the differentiation of 3T3-L1 preadipocytes to adipocytes (day 0–6), the cells were treated with carrier or G and GS as individual compounds and
in combination, and intracellular lipid content was measured and quantified. G (50 µM) and GS (6.25, 12.5 µM) each suppressed lipid accumulation by 40.0 ± 5.1% (P < 0.001), 26.8 ± 1.0% (P < 0.001), and 49.3 ± 1.0% (P < 0.001), respectively, after 6 days of treatment (Fig. 4A). G50+GS6.25 and G50+GS12.5 further decreased lipid accumulation by 83.4 ± 1.4% and 93.9 ± 0.4%, respectively (P < 0.001) more than a calculated additive response (Table 2). Similar results were observed using Oil Red O staining to visualize lipid accumulation in cells after treatments. The representative images show that G50+GS12.5 caused a greater reduction of lipid accumulation when compared to individual compounds (Fig. 4B).

4. Discussion

Guggulsterone has been reported to induce apoptosis [24] in HL60 cells and inhibit differentiation in 3T3-L1 cells [23]. In our previous study, we have demonstrated that genistein decreased body weight in
vivo and induced adipose tissue apoptosis in ovariectomized female mice [15]. Here we report for the first time that G+GS causes enhanced induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells.

This study also investigated the molecular events leading to the induction of apoptosis with G+GS. We have shown that the enhanced apoptotic effects of G+GS are at least partly mediated through the mitochondrial pathway. Recently, mitochondria have been proposed as a novel prospective target for chemotherapy-induced apoptosis [20]. Partial disruption of mitochondrial membrane potential and release of cytochrome c occurs early in apoptosis [16]. In early reports, genistein was shown to induce apoptosis via mitochondrial damage with the involvement of the permeability transition pore (PTP) and caspase-3 activation in T lymphoma cells [3]. Guggulsterone-induced apoptosis was also associated with induction of multidomain pro-apoptotic Bcl-2 family members Bax and Bak in PC-3 human prostate cancer cells [28]. Interestingly, we observed that G+GS decreased pro-caspase-3 (32 kDa) and increased
Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Decrease in Lipid Accumulation (mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.0 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G50</td>
<td>40.0 ± 5.1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>GS6.25</td>
<td>26.8 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>GS12.5</td>
<td>49.3 ± 1.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>G50GS6.25 (calculated)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>66.8 ± 1.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>G50GS12.5 (calculated)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>89.3 ± 1.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G50+GS6.25 (combined)</td>
<td>83.4 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G50+GS12.5 (combined)</td>
<td>93.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>Calculated additive responses.

<sup>a</sup>,<sup>b</sup>,<sup>c</sup>,<sup>d</sup>,<sup>e</sup>,<sup>f</sup>: means without common letters are different, \( P < 0.05 \).

the production of the 17 kDa active caspase-3 cleavage product, while G and GS individually had no effect. Our results also show that apoptosis induced by G+GS was associated with induction of pro-apoptotic Bax and cytochrome c release. Bax exerts pro-apoptotic activity by translocation from the cytosol to the mitochondria, where it induces cytochrome c release [1]. This suggests that the mitochondria may play a key role in the enhancement of apoptosis by G+GS.

Poly(ADP-ribose)polymerase (PARP) is a nuclear enzyme that facilitates DNA repair in response to DNA damage [17]. Activation of caspase 3 cleaves PARP, which is then unable to repair DNA, and consequently the apoptotic process is accelerated [19]. Our data also showed that G+GS induced cleavage of 116-kDa PARP, resulting in the accumulation of an 85-kDa product, while G and GS individually had no effect.

Adipogenesis, the development of mature fat cells from preadipocytes, is an intensely studied model of cellular differentiation in 3T3-L1 cells. Our study showed that treatment of 3T3-L1 cells with G and GS individually during differentiation suppressed lipid accumulation. G+GS further decreased lipid accumulation in a dose-dependent manner. Harmon et al. showed that genistein inhibited the expression of PPAR\(\gamma\) and C/EBP\(\alpha\) in 3T3-L1 cells [8,9], and inhibitory actions of adipocyte differentiation by guggulsterone are mediated through inhibition of the farnesoid X receptor (FXR) [23]. FXR is a member of the nuclear hormone receptor superfamily that was identified as the physiological receptor for bile acid [19]. Exposure of 3T3-L1 cells to a potent and selective FXR ligand increased preadipocyte differentiation and this effect was associated with increased expression of PPAR\(\gamma\)2, C/EBP\(\alpha\), and aP2 mRNAs [23]. GS, which was shown to be a FXR antagonist [33], reversed this effect [23]. We hypothesize that the inhibitory effects of G+GS on adipogenesis might involve suppression of PPAR\(\gamma\) and C/EBP\(\alpha\) expression in 3T3-L1 cells. However, further studies are needed to elucidate the synergistic effects of genistein and guggulsterone (G+GS) on adipogenesis in 3T3-L1 cells.

The concentration range tested in the present study was based on genistein [9,12] and guggulsterone concentrations [23,33] used in other \textit{in vitro} studies. A number of animal studies have shown that oral administration of either gum guggul (3 g/kg body weight in chicks [2]) or purified guggulsterone (25 mg/kg body weight in rats [27]) is effective in decreasing plasma cholesterol levels. However, to our knowledge, measurements of guggulsterone in plasma after oral administration have not yet been reported.

A dose of 150 mg/kg genistein, which results in a plasma genistein concentration of 3.8 ± 0.4 \(\mu\)M [21], when administered to ovariectomized female mice caused weight loss [15,21] and adipose tissue apoptosis. However, at least 100 \(\mu\)M genistein was required to demonstrate a significant increase in apoptosis.
of 3T3-L1 adipocytes in vitro [15]. Thus, these studies indicate the difficulty in making predictions about relationships between concentrations shown effective in vitro under somewhat artificial conditions and effective plasma levels of the same agent.

In conclusion, we demonstrated that although genistein and guggulsterone showed little or no effect as individual compounds, in combination (G+GS) they were more capable of inducing apoptosis and decreasing lipid accumulation in 3T3-L1 adipocytes. Moreover, enhanced effects of the combination on inducing apoptosis are at least partly mediated through the mitochondrial pathway.
Acknowledgements

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References


