Resveratrol Potentiates Genistein’s Antiadipogenic and Proapoptotic Effects in 3T3-L1 Adipocytes 1,2

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Abstract
Genistein (G) and resveratrol (R) individually inhibit adipogenesis in 3T3-L1 adipocytes and induce apoptosis in cancer cells. We investigated whether the combination of G and R resulted in enhanced effects on adipogenesis, lipolysis, and apoptosis in 3T3-L1 cells. Preadipocytes and mature adipocytes were treated with G and R individually at 50 and 100 μmol/L (G100; R100) and in combination. Both in preadipocytes and mature adipocytes, G and R individually decreased cell viability dose-dependently, but G100 + R100 further decreased viability by 59 ± 0.97% (P < 0.001) and 69.7 ± 1.2% (P < 0.001) after 48 h compared with G100 and R100, respectively. G100 + R100 induced apoptosis 242 ± 8.7% (P < 0.001) more than the control after 48 h, whereas G100 and R100 individually increased apoptosis only 46 ± 9.2 and 46 ± 7.9%, respectively. G and R did not modulate mitogen-activated protein kinase expression by themselves, but G100 + R100 increased Jun-N-terminal kinase phosphorylation by 38.8 ± 4.4% (P < 0.001) and decreased extracellular signal-regulating kinase phosphorylation by 48 ± 3.4% (P < 0.001). Individually, G and R at 25 μmol/L (G25; R25) decreased lipid accumulation by 30 ± 1.7% and 20.07 ± 4.27%, respectively (P < 0.001). However, G25 + R25 decreased lipid accumulation by 77.9 ± 3.4% (P < 0.001). Lipolysis assay revealed that neither G25 nor R25 induced lipolysis, whereas G25 + R25 significantly increased lipolysis by 25.5 ± 4.6%. The adipocyte-specific proteins PPARγ and CCAAT/enhancer binding protein-alpha were downregulated after treatment with G + R, but no effect was observed with individual compounds. These results indicate that G and R in combination produce enhanced effects on inhibiting adipogenesis, inducing apoptosis, and promoting lipolysis in 3T3-L1 adipocytes. Thus, the combination of G and R is more potent in exerting antiobesity effects than the individual compounds. J. Nutr. 137: 2668–2673, 2007.

Introduction
Obesity arises from the imbalance between energy intake and energy expenditure, leading to a pathological accumulation of lipids in adipocytes, as well as an increased number of adipocytes. The amount of adipose tissue can be regulated by the inhibition of adipogenesis from precursor cells, preadipocyte and adipocyte apoptosis, as well as adipocyte dedifferentiation (1). Natural products have the potential for inducing apoptosis of adipose tissue, inhibiting bone marrow adipogenesis, and thereby yielding effective treatments for obesity and osteoporosis (2). The biological impact of dietary estrogens on human health has generated considerable interest (3,4), and phytoestrogens, primarily because of their antiproliferative effects, have been the subject of active recent research as anticancer agents.

Relatively little research exists on the effects of phytoestrogens on adipocytes. Genistein (G),5 a soy isoflavone, was shown to decrease food intake, body weight, and fat pad weight in ovariectomized female mice (5,6). In adipocytes, G was shown to inhibit cell proliferation and increase lipolysis (7). In addition to estrogenic effects, G has effects on protein tyrosine kinases, apoptosis, cell proliferation, and angiogenesis (8–10) and can potentially affect adipose tissue through these mechanisms. G was also implicated in cancer control, primarily because of its strong antiproliferative and apoptotic potential (11). Resveratrol (R; 3,5,4′-trihydroxystilbene), a naturally occurring phytoalexin found in red wines and grape juice, was shown to reduce the synthesis of lipids in rat liver (12) and 3T3-L1 adipocytes (13), inhibit the synthesis of eicosanoids in rat leukocytes (14), interfere in arachidonate metabolism (15), and inhibit the activity of some protein kinases (16). R decreased proliferation and induced apoptosis and cell cycle arrest in various cell lines.
Considering the antiadipogenic and lipolytic effects of G and R in murine adipocytes, coupled with their antiproliferative activity in a number of cell lines, we hypothesized that G and R may act synergistically to inhibit the signals that promote adipogenesis and decrease adipose tissue mass by apoptosis.

Interaction among the members of the C/EBP and PPAR families plays an important role in the adipogenesis process. CCAAT/enhancer binding protein-beta (C/EBPβ) is expressed immediately after the induction of differentiation, and then PPARγ and CCAAT/enhancer binding protein-alpha (C/EBPα) act synergistically to promote adipogenesis (20–22). Harmon et al. (23,24) showed that G inhibited the expression of PPARγ and C/EBP in 3T3-L1 cells. However, the effect of R on PPARγ and C/EBPα expression is not known. In this study, we predicted that G and R would inhibit adipogenesis by modulating the expression of C/EBPα and PPARγ.

Given that phytoestrogens inhibit proliferation of several cell lines (25,26), we investigated the combination effect of G and R on adipocyte apoptosis. Mitogen-activated protein kinase (MAPK) pathways regulate diverse cellular activities, including cell survival, apoptosis, and differentiation. The MAPK pathways consist of 3 parallel kinase modules, that is, the extracellular signal-regulating kinase (ERK1/2), the Jun-N-terminal kinase (JNK), and the p38 MAPK pathways. In general, JNK and p38 MAPK activation is associated with apoptosis induction (27), whereas ERK1/2 are preferentially activated by phorbol esters (28) and are cytoprotective (27). R downregulated MAPK/JNK/p38 in the vasculature (29). G was shown to decrease ERK1/2 phosphorylation in various cell lines (30). In this study, we predicted that G and R would induce apoptosis by modulating ERK1/2 and JNK pathways.

Our objective was to examine the possibility of interaction between G and R, resulting in enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes.

Materials and Methods

Cell line and cell culture. 3T3-L1 mouse embryonic fibroblasts were obtained from American Type Culture Collection and were cultured as described elsewhere (31). Briefly, cells were cultured in DMEM containing 10% bovine calf serum until confluent. Two days after confluence (d 0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 167 nmol/L insulin, 0.5 μmol/L 3-isobutyl-1-methylxanthine, and 1 μmol/L dexamethasone for 2 d. On d 2, differentiation medium was replaced with 10% FBS/DMEM medium containing 167 nmol/L insulin and incubated for 2 d, followed by culturing with 10% FBS/DMEM medium for an additional 4 d, at which time >90% of the cells were mature adipocytes with accumulated fat droplets. All media contained 1% penicillin-streptomycin (10,000 units/mL and 1% (v/v) 100 mmol/L pyruvate. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

Cell viability and apoptosis assays. Tests were performed in 96-well plates. For mature adipocytes, cells were seeded (5000 cells/well) and grown to maturation as described above. For preadipocytes, cells were seeded (2500 cells/well) and assay performed 3 d after seeding. Preadipocytes or mature adipocytes were incubated with either 0.2% dimethyl sulfoxide (DMSO) or test compounds for 24 and 48 h. Cell viability assay was performed per the manufacturer’s instructions. The absorbance was measured at 490 nm in a plate reader (μQuant, Bio-Tek Instruments) to determine the formazan concentration, which is proportional to the number of live cells. For measuring the extent of apoptosis, ApoStrand ELISA apoptosis detection kit was used. Cells were grown in 96-well plates, treated with test compounds for 24 and 48 h, and assayed as per the manufacturer’s instructions. The assay selectively detects single-stranded DNA, which occurs in apoptotic cells but not in necrotic cells or cells with DNA breaks in the absence of apoptosis (32). Assays were performed at least 2 times with 6 replicates for each treatment.

Quantification of lipid content. Lipid content was quantified using commercially available AdipoRed assay reagent. In brief, postconfluent preadipocytes grown in 96-well plates were incubated with 0.2% DMSO or test compounds during the adipogenic phase, and on d 6, cells were assayed for lipid content according to the manufacturer’s instructions. The experiments were performed with at least 6 replicates per treatment and repeated 3 times.

Lipolysis assay. To determine the extent of lipolysis induced by test compounds, mature adipocytes were treated with either 0.2% DMSO or test compounds for 5 h, and the free glycerol released was assayed by using a Lipolysis assay kit for 3T3-L1 adipocytes (Zen-Bio) and following the manufacturer’s instructions. The experiment was repeated 2 times with at least 4 replicates.

Western blot analysis. Mature adipocytes were treated with 100 μmol/L each of G and R as individual compounds and in combination for 3 h. Control cells were treated with 0.2% DMSO. Likewise, maturing preadipocytes were treated with 25 μmol/L each of G and R alone and in combination for 6 d. Whole cell extracts were prepared as described elsewhere (33). The protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as the standard. Western blot analysis was performed using the commercial NUPAGE system (Novex/Invitrogen), 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, separated by 12% acrylamide gels, and analyzed by immunoblotting, as previously described (34).

Quantitative analysis of Western blot data. Measurement of signal intensity on polyvinylidene fluoride membranes after Western blotting with various antibodies was performed using a FluorChem densitometer with the AlphaEaseFC image processing and analysis software (Alpha Innotech). For statistical analysis, all data were expressed as integrated density values, which were calculated as the density values of the specific protein bands/β-actin density values and expressed as a percentage of the control. All figures showing quantitative analysis include data from at least 3 independent experiments.

Reagents. PBS and DMEM were purchased from Gibco (BRL Life Technologies). ApoStrand ELISA apoptosis detection kit was purchased from BiOMOL. The viability assay kit (CellTiter 96 Aqueous one solution cell proliferation assay) was purchased from Promega. R was from Sigma. AdipoRed Assay Reagent was from Cambrex BioScience. G (99% pure) was purchased from Indofine Chemical Company. Antibodies specific for β-actin, C/EBPα, C/EBPβ, and PPARγ were purchased from Santa Cruz Biotechnology. Antibodies for phospho-JNK (Thr183/Yhr185), total JNK, phospho-ERK1/2 (Thr202/Tyr204), and total ERK1/2 were from Cell Signaling Technology.

Statistical analysis. ANOVA (GLM procedure, Statistica, version 6.1; StatSoft) was used to determine the significance of treatment and time effects and interactions (time vs. treatment). Fisher post-hoc least significant difference test was used to determine the significance of differences among means. In some cases, to estimate differences among 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 ± SE.

Results

Cell viability of preadipocytes and mature adipocytes. G and R as individual compounds decreased cell viability in preadipocytes, and the combinations, G at 50 μmol/L (G50) + R at 50 μmol/L (R50) and G at 100 μmol/L (G100) + R at 100 μmol/L (R100), further decreased cell viability by 38 ± 0.89%
48-h treatment (Table 1). The percentage decrease in viability, based on a calculated additive response to G100 + R100 after 48 h, was 55.2 ± 1.49%, which is not significantly (P = 0.389) different from the combined treatment. Similarly, mature adipocytes were treated with G50 and R50, as individual compounds and in combination (G50 + R50 and G100 + R100), for 24 and 48 h. G and R individually decreased cell viability, and the combination G100 + R100 further decreased cell viability by 41.7 ± 2.1% (P < 0.001) after 24 h and 69.7 ± 1.2% (P < 0.001) after 48 h (Table 1). However, the percentage decrease in viability, based on the calculated additive treatment after 48 h, was only 43.53 ± 4.73%, which is less than the combined treatment effect (P < 0.001). G100 and R100 were selected for subsequent apoptosis experiments.

**Induction of apoptosis in mature adipocytes.** Neither G50 nor R50, as individual treatments, increased apoptosis, but at 100 μmol/L, both compounds increased apoptosis by 50% (Table 2). However, exposure of mature adipocytes to G50 + R50 and G100 + R100 combinations resulted in an enhanced increase of cell death. G100 + R100 treatment increased apoptosis 146 ± 6.8% (P < 0.001) and 242 ± 8.7% (P < 0.001) more than the control after 24- and 48-h incubation periods, respectively. The calculated additive effect of G100 + R100 after 48 h was only 93.0 ± 17.1%.

**Modulation of MAPK levels.** Both G and R were shown to modulate MAPK levels in other cell lines. To determine whether the apoptosis induced by these 2 compounds was related to changes in MAPK levels, we investigated the effect of individual compounds and combinations on ERK1/2 and JNK levels. Changes in MAPK levels, we investigated the effect of individual compounds and combinations on ERK1/2 and JNK levels. To determine whether the apoptosis induced by these 2 compounds was related to changes in MAPK levels, we investigated the effect of individual compounds and combinations on ERK1/2 and JNK levels. Incubation of mature adipocytes with DMSO, G100, R100, or G100 + R100 for 3 h led to enhanced phosphorylation of JNK with the combination treatment only (Fig. 1A). Quantitative analysis showed that neither G100 nor R100 affected JNK phosphorylation, but the combination of G100 and R100 each increased the phosphorylation by −38.8 ± 4.4% (P < 0.001). Incubation of mature adipocytes for 3 h with G100 + R100 decreased ERK1/2 phosphorylation by 48 ± 3.4% (P < 0.001; Fig. 1B). R and G alone had no effect.

**Inhibition of lipid accumulation.** In maturing preadipocytes, preliminary experiments with a range of G and R concentrations (data not included) showed that the combined effect on adipogenesis was very potent. Therefore, lower concentrations were used in experiments with maturing preadipocytes. G25 and R25, as individual compounds, decreased lipid accumulation by 30 ± 1.7% and 20.07 ± 4.27%, respectively (P < 0.001), but did not affect viability. The G25 + R25 combination decreased lipid accumulation by 77 ± 3.4% (P < 0.001), whereas the calculated additive response of G25 + R25 was a decrease in lipid accumulation by 49.8 ± 3.8% (different from the combination treatment, P < 0.0001; Fig. 2A). Even though the G25 + R25 combination enhanced effects on viability in maturing preadipocytes (24 ± 1.8% decrease, P < 0.01; Fig. 2B), the effect on adipogenesis was more potent than the effect on viability, indicating a true decrease in lipid accumulation.

**Effects on lipolysis.** Neither G25 nor R25 induced lipolysis, whereas G25 + R25 increased it by 25.5 ± 4.6% (P < 0.01).

**Effects on PPARγ, C/EBPa, and C/EBPβ expression.** To determine whether the decrease in lipid accumulation with G and R combinations was related to C/EBPβ, C/EBPa, and PPARγ expression levels, whole cell lysates were extracted after treatment, as described previously, and subjected to Western blotting using anti-C/EBPβ, anti-C/EBPa, anti-PPARγ, and anti-β-actin antibodies. Quantitative analysis revealed that neither G25 nor R25 altered the expression levels of C/EBPa and PPARγ, whereas the combination (G25 + R25) decreased the expression levels of C/EBPa by 56 ± 5.1% (P < 0.001; Fig. 3B) and PPARγ

**Table 1** Percent change in viability in 3T3-L1 preadipocytes and mature adipocytes treated with G50 or G100 plus R50 or R100 for 24 and 48 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preadipocyte 24 h</th>
<th>Mature adipocyte 24 h</th>
<th>Preadipocyte 48 h</th>
<th>Mature adipocyte 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 0.2% DMSO</td>
<td>0.00 ± 4.0%ab</td>
<td>0.00 ± 2.0%ab</td>
<td>0.00 ± 1.5%ab</td>
<td>0.00 ± 0.6%ab</td>
</tr>
<tr>
<td>G50</td>
<td>7.30 ± 3.6%a</td>
<td>1.76 ± 1.6%ab</td>
<td>5.81 ± 2.6%a</td>
<td>-6.99 ± 2.5%a</td>
</tr>
<tr>
<td>R50</td>
<td>-6.60 ± 3.5%b</td>
<td>-1.57 ± 1.5%ab</td>
<td>-8.65 ± 2.6%b</td>
<td>-22.55 ± 2.1%b</td>
</tr>
<tr>
<td>R100</td>
<td>-4.29 ± 4.0%bc</td>
<td>-21.14 ± 1.2%cd</td>
<td>-8.36 ± 2.5%bc</td>
<td>-10.85 ± 2.1%bc</td>
</tr>
<tr>
<td>G50 + R50 (Combined)</td>
<td>-25.84 ± 2.9%a</td>
<td>-33.66 ± 0.9%ab</td>
<td>-17.41 ± 2.6%a</td>
<td>-20.98 ± 3.0%a</td>
</tr>
<tr>
<td>G100 + R100 (Combined)</td>
<td>-29.42 ± 0.9%a</td>
<td>-37.25 ± 0.8%ab</td>
<td>-11.61 ± 2.0%a</td>
<td>-8.50 ± 2.1%a</td>
</tr>
<tr>
<td>G50R50 (Calculated)*</td>
<td>-48.41 ± 1.1%a</td>
<td>-58.14 ± 0.9%bc</td>
<td>-41.60 ± 2.0%b</td>
<td>-69.30 ± 1.2%b</td>
</tr>
<tr>
<td>G100R100 (Calculated)*</td>
<td>-3.01 ± 3.6%bc</td>
<td>-19.37 ± 1.6%cd</td>
<td>-2.54 ± 1.5%bc</td>
<td>-17.84 ± 4.4%cd</td>
</tr>
</tbody>
</table>

* Values are means ± SEM, n = 6. Within a cell stage, means without a common letter differ, P < 0.05.
* Calculated additive responses.

![Image](https://example.com/image.png)
by 48 ± 4.4% (P < 0.05; Fig. 3C), respectively. However, none of the treatments affected C/EBPβ expression levels.

**Discussion**

In this study, we describe how flavonoids, like G and R, in combination can exert an enhanced effect on inducing apoptosis and inhibiting adipogenesis in 3T3-L1 adipocytes. The decrease in the mature adipocyte number and size was shown to involve the loss of lipids through lipolysis and the loss of cells through apoptosis (1,35). We also performed an investigation aimed at delineating the molecular events that might be partially involved in the blockade of adipogenesis and the induction of apoptosis.

The relationship among dietary flavonoids and weight loss has not been explored adequately. However, in vivo studies suggest that isoflavones may be useful in the treatment of obesity. Isoflavone-rich diets improved lipid metabolism and had antidiabetic effects in obese rats (36). G, an isoflavone, was also shown to have direct effects on lipid metabolism in the liver and adipose tissue, decreasing triglycerides while increasing FFA in serum (37). Consistent with these results, in our study, G decreased lipid accumulation by ~18% in maturing 3T3-L1 adipocytes, even at concentrations as low as 25 μmol/L. It was already demonstrated that apoptosis was a contributing factor to G’s reducing effect on body weight (5), and in our study, G100 induced apoptosis by ~50% more than the control. R also inhibited adipogenesis by ~45% at 25 μmol/L, as shown previously (13). In addition, R induced apoptosis in mature adipocytes. Both of these flavonoids were effective by themselves at higher concentrations in inducing apoptosis, but not at 50 μmol/L. However, the effect of the combination (G50 + R50) in inducing apoptosis was not different from either G100 or R100 alone, although it was different from the calculated additive response (G50R50; Table 2). Likewise, the G100 + R100 combination had a greater effect than the calculated additive response (G100R100). Thus, based on limited dose testing, it is difficult to know whether the apoptotic effect was synergistic or additive. In contrast, G25 + R25 showed a much greater response in inhibiting adipogenesis than either G50 or R50, indicating that the combination effect is more than additive.

To elucidate the mechanism of apoptosis induced by G and R, we studied ERK1/2 and JNK expression. We found that G
and R individually did not significantly alter ERK1/2 or JNK levels. However, in combination, they decreased ERK1/2 phosphorylation by ~50% and increased JNK phosphorylation by 40%. ERK1/2 activation, in general, is considered to be cytoprotective, and JNK activation was shown to be associated with apoptosis induction (27). Though ERK1/2 activation results in cell proliferation (38), a few studies showed that, depending on the cell type, ERK1/2 activation may also result in cell death (39,40). Our finding that G did not activate JNK is in agreement with the finding that G increased the activity of the JNK pathway in A431 cells (41), but it is in agreement with the finding that G did not activate JNK in MCF-10F cells (42). Inconsistent with our results, G inactivated ERK1/2 in cell lysates from the treated cells were collected on d 6 after induction, we did not expect to see altered expression levels of C/EBPβ because it is an early adipogenic marker. However, G and R in combination blocked differentiation by significantly suppressing the upregulation of both PPARγ and C/EBPα, correlating with results from the AdipoRed assay.

In addition to antiadipogenic effects, we also investigated the effects of G and R on lipolysis. G100 for 24 h induced a 6-fold greater release of glycerol into the culture medium than did the control in 3T3-L1 cells (23). In a different study, upregulation of Sirt1 by R was shown to trigger lipolysis in 3T3-L1 cells (13). These results are not in agreement with our findings that neither G25 nor R25 increased lipolysis. Differences in dose and incubation periods might contribute to the varied responses of these compounds. However, the combination of G and R significantly increased lipolysis, indicating that the antiadipogenic effect of this combination is at least partially mediated via enhancement of lipolysis.

The polyphenolic compounds present in fruits and vegetables regulate cell proliferation and induce apoptosis (48). R and quercetin, a flavonoid, synergistically induced apoptosis in human leukemia cells (49). Similarly, G and thearubigins (a flavone obtained from black tea) synergistically inhibited growth of prostate tumor cells. In this study, we showed that G and R synergistically inhibited adipogenesis and induced apoptosis in 3T3-L1 adipocytes. Although results from in vitro experiments cannot be directly extrapolated to clinical effects, these studies may help in elucidating various molecular pathways involved in the overall disease process of obesity. Moreover, a dose of 150 mg/kg G administered to ovariectomized female mice caused weight loss (5,6) and adipose tissue apoptosis (5), whereas 100 μmol/L G was the minimum concentration required to demonstrate a significant increase in apoptosis of 3T3-L1 adipocytes in vitro after 24 h (5). This is interesting because a dose of 150 mg/kg G in mice resulted in a plasma G concentration of 3.8 ± 0.4 μmol/L (6).

Thus, these studies reflect the difficulty in making predictions about relationships among concentrations of agents that are shown to be effective in vitro under somewhat artificial conditions and effective plasma levels. To summarize, we demonstrated that G and R at tested concentrations are not very effective as individual compounds, but in combination, they are more capable of inducing apoptosis and decreasing lipid accumulation in adipocytes.

**Literature Cited**


