Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis

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Harmon, Anne W., and Joyce B. Harp. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. Am J Physiol Cell Physiol 280: C807–C813, 2001.—Flavonoids, polyphenolic compounds that exist widely in plants, inhibit cell proliferation and increase cell differentiation in many cancerous and noncancerous cell lines. Because terminal differentiation of preadipocytes to adipocytes depends on proliferation of both pre- and postconfluent preadipocytes, we predicted that flavonoids would inhibit adipogenesis in the 3T3-L1 preadipocyte cell line. The flavonoids genistein and naringenin inhibited proliferation of preconfluent preadipocytes in a time- and dose-dependent manner. When added to 2-day postconfluent preadipocytes at the induction of differentiation, genistein inhibited mitotic clonal expansion, triglyceride accumulation, and peroxisome proliferator-activated receptor-γ expression, but naringenin had no effect. The antiadipogenic effect of genistein was not due to inhibition of insulin receptor substrate-1 tyrosine phosphorylation. When added 3 days after induction of differentiation, neither flavonoid inhibited differentiation. In fully differentiated adipocytes, genistein increased basal and epinephrine-induced lipolysis, but naringenin had no significant effects. These data demonstrate that genistein and naringenin, despite structural similarity, have differential effects on adipogenesis and adipocyte lipid metabolism.

Although relatively little research exists on the effects of flavonoids on adipose cells, Kuppusamy and Das (14, 15) have found that several flavonoids potentiate epinephrine-induced lipolysis in primary rat adipocytes. Shisheva and Shechter (30) found that quercetin, a flavanol structurally similar to both genistein and naringenin, blocks insulin-mediated lipogenesis by preventing the insulin receptor tyrosine kinase from phosphorylating substrate. These potentially lipolytic and antilipogenic effects in rat adipocytes, coupled with antiproliferative activity in a number of cell lines, suggest that flavonoids may decrease adipose tissue mass or inhibit the signals that promote adipogenesis.

Given that flavonoids inhibit proliferation not only of cancer cells but also of normal cells, such as mammary (20) and intestinal (7) epithelial cells, we hypothesized that flavonoids would inhibit proliferation of preadipocytes. Because terminal differentiation to adipocytes depends on proliferation of both pre- and postconfluent preadipocytes (18), we predicted that flavonoids would also inhibit the differentiation process.

To test the effects of flavonoids on preadipocytes, we used murine 3T3-L1 cells. This cell line allows investigation of stimuli and mechanisms that regulate preadipocyte replication separately from those that regulate differentiation to adipocytes. As preadipocytes, these cells have a fibroblastic appearance. They replicate in culture until they form a confluent monolayer, after which cell-cell contact triggers G1/G0 growth arrest (9). Subsequent stimulation with 3-isobutyl-1-methylxanthine, dexamethasone, and high doses of insulin (MDI) for 2 days prompts these cells to undergo postconfluent mitotic clonal expansion, exit the cell cycle, and begin to express adipocyte-specific genes (25). Approximately five days after induction of differentiation, >90% of the cells display the characteristic lipid-filled adipocyte phenotype.

In this report, we showed that in 3T3-L1 cells, genistein and naringenin inhibited proliferation of postconfluent preadipocytes. Although genistein inhibited MDI-induced proliferation of postconfluent preadipocytes and their subsequent differentiation into adipocytes, naringenin had no effect on these processes. In
mature adipocytes, genistein strongly induced lipolysis, both alone and in combination with epinephrine. Naringenin did not produce a statistically significant increase in lipolysis. These findings in cultured adipose cells suggest that dietary flavonoids, particularly genistein, may have inhibitory effects on adipose tissue enlargement in vivo.

**MATERIALS AND METHODS**

**Materials.** Genistein (4’,5,7-trihydroxyisoflavone) and all tissue culture materials were from Gibco (Grand Island, NY). Naringenin (4’,5,7-trihydroxyflavanone), epinephrine, insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were from Sigma (St. Louis, MO). Rabbit polyclonal anti-insulin receptor transducers and activators of transcription (STAT) 3 antibody was from New England Biolabs (Beverly, MA). Mouse monoclonal anti-peroxisome proliferator-activated receptor (PPAR)-γ (E-8), rabbit polyclonal anti-insulin receptor substrate (IRS)-1 (C-20) antibodies, and protein A/G plus agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody (PY29) was from Pharmingen-Transduction Laboratories (Lexington, KY). The enhanced chemiluminescence (ECL) detection kit and horseradish peroxidase-conjugated secondary antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ).

**Cell culture.** 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were cultured in DMEM with 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) penicillin-streptomycin (10,000 U/ml penicillin and 10,000 μg/ml streptomycin in 0.85% saline), and 1% (vol/vol) 100 mM sodium pyruvate at 37°C in 95% air–5% CO2. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM dexamethasone, and 10 μg/ml insulin (MDI) added to DMEM/10% FBS culture medium. On day 3, the MDI medium was replaced with DMEM/10% FBS, which was changed every 2 days thereafter until analysis on days 7–10. Flavonoids were reconstituted as 100 mM stock solutions in DMSO, filter sterilized, and stored at 20°C. For each experiment, cells received flavonoids premixed with culture medium. Unless otherwise noted, “vehicle” refers to 0.1% DMSO in culture medium or MDI differentiation medium.

**Proliferation assay.** Preconfluent 3T3-L1 preadipocytes were seeded in 96-well plates at a density of 10,000 cells/100 μl/well. Vehicle or flavonoids, in doses ranging from 1 to 100 μM, were added to culture medium with or without cells at the time of plating. At 0, 12, 24, and 48 h after plating, a colorimetric proliferation assay (CellTiter 96 AQueous nonradioactive cell proliferation assay; Promega, Madison, WI) was performed as directed by the manufacturer. The percent lactate dehydrogenase (LDH) release was calculated as follows: % LDH release = (LDH in culture supernatant/LDH in culture supernatant + LDH in cell lysate) × 100.

**Triglyceride assay.** On days 7–10 after differentiation, 3T3-L1 cells were washed with PBS, scraped on ice in 100 μl of saline solution (2 M NaCl, 2 mM EDTA, 50 mM sodium phosphate, pH 7.4), sonicated to homogenize the cell suspension, and assayed for total triglyceride (GPO-Trinder; Sigma) according to the method of Shimabukuro et al. (29). Results were expressed as total triglyceride per cellular protein (DC protein assay; Bio-Rad, Hercules, CA). For lipolysis experiments, the conditioned medium was assayed for glycerol content 24 h after treatment (GPO-Trinder; Sigma).

**Oil Red O staining.** 3T3-L1 adipocytes were washed with PBS, fixed with 10% glutaraldehyde in PBS (pH 7.4), then stained with 0.5% Oil Red O.

**Immunoblot analysis.** 3T3-L1 cells were washed twice in ice-cold PBS with 1 mM orthovanadate, then placed immediately in sample buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% Na3VO4, 10 mg/ml aprotinin, 1 mM pepstatin, 16.4 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM Na2VO4, 2% SDS, and 10% glycerol] without dithiothreitol (DTT) or tracking dye. Lysates were heated and protein concentrations determined before adding 100 mM DTT and tracking dye. Protein concentrations were determined in cell lysates using the Bio-Rad DC protein determination kit. Bovine serum albumin (BSA) was used as a standard. Samples were heated for 5 min at 95°C, separated by 10% SDS-PAGE, and analyzed by immunoblotting as previously described (16, 19). Immunoblots were developed with the ECL kit.

**Immunoprecipitation.** Cells were plated in ice-cold RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM Na3VO4, 1 mM PMSF, and 10 μg/ml aprotinin) and rocked on ice for 30 min. The lysed cells were harvested, centrifuged at 7,000 g for 20 min at 4°C, supernatants were removed, and protein concentrations determined using the Bio-Rad DC protein determination kit, with BSA used as a standard for immunoprecipitations. 2.5–10 μg/ml of PY29 antibodies were added to lysates, which contained equal amounts of protein (200–300 μg); incubation occurred overnight at 4°C. Protein A/G plus agarose beads were added and agitated for 1 h at 4°C. Immunoprecipitates were recovered by centrifugation at 2,500 g and washed three times with ice-cold RIPA lysis buffer. Immunoprecipitated proteins were dissolved in 2× sample buffer and analyzed as described above.

**Statistical analysis.** Data are expressed as means ± SE. The significance of differences between means, set at P < 0.05, was assessed by one-way analysis of variance and Bonferroni post hoc testing (NCSS Statistical Software, Kaysville, UT).

**RESULTS**

Flavonoids inhibit proliferation of preconfluent preadipocytes. To determine whether flavonoids inhibit the proliferation of preadipocytes, we cultured preconfluent 3T3-L1 preadipocytes in the presence and absence of varying doses of genistein or naringenin, then measured cell proliferation at several points during the course of growth. As shown in Fig. 1, the addition of genistein or naringenin at the time of plating inhibited proliferation of 3T3-L1 preadipocytes in a dose- and time-dependent manner. At 48 h, 100 μM genistein...
inhibited proliferation by nearly 60%; similarly, 100 μM of naringenin reduced proliferation by 40%.

To determine whether flavonoids reduced proliferation through cytotoxic effects, we measured LDH release into the culture medium in response to flavonoid treatment. Figure 2 shows that 100 μM of genistein produced no cytotoxic effects. Although all cells released more LDH over time, roughly 60% more at 48 h than at 24 h, 100 μM of naringenin triggered significantly more LDH release at both time points than did the control (vehicle, 0.1% DMSO).

Genistein inhibits adipogenesis. We next examined the effects of flavonoids on preadipocyte differentiation. Given the effects of flavonoids on postconfluent mitotic clonal expansion, we hypothesized that genistein inhibited postconfluent mitotic clonal expansion by roughly 30% (Fig. 3), naringenin had no effect on this process. As shown in Fig. 4, neither genistein nor naringenin triggered significantly more LDH release than did control cells during postconfluent mitotic clonal expansion, indicating that neither flavonoid produced significant cytotoxic effects at this stage.

**Fig. 1. Effect of flavonoids on 3T3-L1 preadipocyte proliferation.** Genistein (A) and naringenin (B) were added to preconfluent preadipocytes in various concentrations (vehicle, 5 μM, 50 μM, and 100 μM) at the time of plating. Cells were maintained until the degree of proliferation, expressed as percent of control (vehicle), was determined via a colorimetric proliferation assay (0, 12, 24, and 48 h after plating). Data shown reflect the means ± SE of 9–10 experiments. Within each time point, different letters (a, b, c) indicate statistically significant differences between means at P < 0.05.

**Fig. 2. Cytotoxicity of flavonoids on preconfluent 3T3-L1 preadipocytes.** Preconfluent 3T3-L1 preadipocytes were treated with 100-μM doses of flavonoids or vehicle at the time of plating. After 24 or 48 h, lactate dehydrogenase (LDH) was measured in supernatants and cell lysates. Cytotoxicity was expressed as % LDH release. Data shown reflect the means ± SE of 3 experiments. Within each time point, different letters (a, b) indicate statistically significant differences between means at P < 0.05.

**Fig. 3. Effect of flavonoids on differentiation-induced mitotic clonal expansion of postconfluent 3T3-L1 preadipocytes.** Two-day postconfluent 3T3-L1 cells were differentiated according to the MDI protocol plus 100 μM of flavonoids or vehicle for 72 h. The degree of postconfluent mitotic clonal expansion, expressed as percent of control, was determined via a colorimetric proliferation assay at 0 and 72 h after MDI. Data shown reflect the means ± SE of 3 experiments. Within each time point, different letters (a, b) indicate statistically significant differences between means at P < 0.05.
would inhibit differentiation but that naringenin would not. We treated 2-day postconfluent preadipocytes with MDI plus 100 μM flavonoids or vehicle for 72 h. From day 3 to days 7–9 after induction of differentiation, we maintained cells in culture medium without flavonoids. Cells treated with MDI plus 100 μM genistein accumulated roughly 40% of the intracellular triglyceride contained in controls, as shown by Oil Red O staining (Fig. 5A) and triglyceride accumulation (Fig. 5B). Naringenin did not inhibit triglyceride accumulation. We also determined the effects of flavonoids or vehicle on the expression of PPAR-γ, a nuclear hormone receptor that regulates adipogenesis and is expressed during differentiation (32). Western blot analysis showed that vehicle- and naringenin-treated cells expressed PPAR-γ strongly and equally (Fig. 5C), whereas genistein-treated cells expressed considerably less PPAR-γ. The expression of STAT3, which does not change during proliferation and differentiation of 3T3-L1 cells (8), was unchanged by flavonoid treatment.

Because preadipocytes express low levels of insulin receptor but high levels of insulin-like growth factor I (IGF-I) receptor, insulin appears to signal through the IGF-I receptor at the induction of differentiation (31). Both the IGF-I receptor and insulin receptor undergo autophosphorylation in response to ligand binding and subsequently phosphorylate tyrosine residues on a common substrate, IRS-1. To determine whether genistein, a potent tyrosine kinase inhibitor (2), blocks adipogenesis by inhibiting insulin-stimulated receptor tyrosine kinase activity, we stimulated 2-day postconfluent 3T3-L1 cells with 10 μg/ml insulin in the presence of flavonoids or vehicle. As shown in Fig. 6, neither flavonoid inhibited insulin- or MDI-induced tyrosine phosphorylation of IRS-1. However, genistein was able to inhibit the tyrosine phosphorylation of other proteins in these cells (data not shown).

Given that genistein inhibited both postconfluent mitotic clonal expansion and differentiation to adipocytes, we next considered whether flavonoids would inhibit differentiation when added after mitotic clonal expansion but before triglyceride accumulation. Treating 3T3-L1 cells with 100 μM of flavonoids from days 3–5 did not alter adipogenesis compared with controls (Fig. 7).

Genistein induces adipocyte lipolysis. Genistein and naringenin both potentiate epinephrine-induced lipolysis in primary rat adipocytes (14). To determine whether flavonoids exert similar lipolytic effects in 3T3-L1 cells, we treated 3T3-L1 adipocytes (days 8–10 after MDI stimulation) with 100 μM of flavonoids or vehicle with or without 0.1 μM or 1 μM epinephrine. As shown in Table 1, genistein alone induced a sixfold greater release of glycerol into the culture medium than did control (vehicle). The combination of genistein and epinephrine produced an additive effect: a two- to fourfold increase over epinephrine, depending on the epinephrine concentration. Naringenin had no significant effects on lipolysis.

DISCUSSION

In this report, we found that genistein inhibited the proliferation of both preconfluent and postconfluent preadipocytes. In contrast, naringenin exerted significant dose-dependent antiproliferative effects only at the preconfluent stage. Naringenin did not inhibit mitotic clonal expansion of 2-day postconfluent 3T3-L1 preadipocytes, nor did it inhibit their differentiation to adipocytes. Genistein, on the other hand, blocked differentiation but only when administered at the onset of differentiation. Exposing 3T3-L1 cells to genistein from days 3–5 after induction of differentiation did not alter adipogenesis. These findings demonstrate that genistein’s antiadipogenic effects occurred during the first 72 h after induction of differentiation. These data are consistent with studies showing the occurrence of two critical events during this 72-h period: mitotic clonal expansion and an irreversible commitment to differentiation (11, 28).

Genistein did not prevent insulin-stimulated tyrosine phosphorylation of IRS-1. This finding suggests that genistein exerts its antidiifferentiation effects independently of insulin-stimulated activation of IRS-1. Genistein may block adipogenesis by inhibiting other receptor or nonreceptor tyrosine kinases activated by MDI during differentiation. Alternatively, genistein may inhibit the effects of insulin or other inducers of differentiation independently of tyrosine kinase activity. In isolated rat adipocytes, genistein suppresses the antilipolytic effects of insulin and inhibits insulin-stimulated glucose oxidation with 50% inhibitory dose values of 25 and 15 μg/ml, respectively. Genistein exerts these inhibitory effects without preventing autophosphorylation of the insulin receptor or its subsequent tyrosine phosphorylation of substrate (1).

When investigating the effects of the tyrosine kinase Syk on 3T3-L1 adipogenesis, Wang and Malbon (33) found that genistein exerts a dose-responsive antiadipogenic effect. Consistent with our results, they found that 100 μM genistein potently blocks differentiation. They also demonstrated that a 10-μM dose reduces 3T3-L1 adipogenesis. However, in that report, they did not determine whether genistein inhibits Syk activity.
In addition to its antiadipogenic effects, we also found that 100 μM of genistein, alone and in combination with epinephrine, strongly promoted lipolysis when administered to mature 3T3-L1 adipocytes. Naringenin did not have a statistically significant lipolytic effect. These findings differ from those of Kuppusamy and Das (14), who observed that in isolated rat adipocytes, 250 μM of naringenin promotes epinephrine-
induced lipolysis to a greater degree than does a comparable dose of genistein (68 vs. 31% increase over control, respectively). Although the use of different cell systems may contribute to the varying results between studies, the differences in flavonoid doses may account for much of the discrepancy. Kandulska et al. (12) found that in isolated rat adipocytes, 1 mM of genistein (G), 100 μM naringenin (N), or vehicle (V) for 30 min, then were stimulated with 10 μg/ml insulin and fresh flavonoids/vehicle for 5 or 30 min. Insulin was also added to cells for 2 min in the absence of vehicle as a positive control (A; n = 2). Cells were also stimulated with MDI plus flavonoids or vehicle for 5 and 30 min (B; n = 2). Phosphotyrosine-containing proteins were immunoprecipitated (IP) from cell lysates with 2.5–10 μg/ml anti-phosphotyrosine antibody (PY20). The immunoprecipitated proteins were analyzed by 10% SDS-PAGE and blotted with anti-IRS-1 antibody.

Table 1. Effect of flavonoids on 3T3-L1 adipocyte lipolysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Epinephrine Concentration, μM</th>
<th>Percent of Control for Glycerol Released Per Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Genistein (G)</td>
<td>0</td>
<td>562 ± 22.8*</td>
</tr>
<tr>
<td>Naringenin (N)</td>
<td>0</td>
<td>115 ± 10.7</td>
</tr>
<tr>
<td>Epinephrine (E)</td>
<td>0.1</td>
<td>180 ± 17.3</td>
</tr>
<tr>
<td>G + E</td>
<td>0.1</td>
<td>804 ± 42.5†</td>
</tr>
<tr>
<td>N + E</td>
<td>0.1</td>
<td>269 ± 30.5</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>427 ± 11.0</td>
</tr>
<tr>
<td>G + E</td>
<td>1</td>
<td>920 ± 123.0‡</td>
</tr>
<tr>
<td>N + E</td>
<td>1</td>
<td>666 ± 60.0</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 experiments and are expressed as percent control for glycerol released per well. Two-day postconfluent 3T3-L1 cells were differentiated according to the MDI protocol. Fully differentiated cells were treated for 24 h with vehicle or 100 μM flavonoids ± 0.1 or 1 μM epinephrine. The conditioned medium was then removed from each well and assayed for glycerol content. *P < 0.05, significantly different from vehicle; †P < 0.05, significantly different from 0.1 μM epinephrine; ‡P < 0.05, significantly different from 1 μM epinephrine.

promote loss of body fat. According to Barnes et al. (4), the average Asian’s intake of genistein ranges from 20–80 mg/day, yet the average American consumes only 1–3 mg/day. With high consumption of soyfoods, tissue concentrations of genistein typically do not exceed the 1–5 μM range (5). However, a nontoxic pharmacological dose of genistein, 8 mg/kg body wt, elevates serum genistein to the 10-μM range (personal communication from S. Zeisel, Univ. of North Carolina at Chapel Hill), a level sufficient to inhibit adipogenesis in 3T3-L1 cells (33).

The few existing animal studies that have evaluated the effects of genistein treatment on lipid metabolism or body weight support our findings in 3T3-L1 cells. Nogowski et al. (23) examined the effects of genistein on the lipid metabolism of ovariectomized rats and found that dietary genistein significantly decreases triglyceride levels in serum and muscle but increases the free fatty acid concentration in serum. After incubating isolated adipocytes with genistein, they found inhibited basal and insulin-induced lipogenesis as well as enhanced epinephrine-induced lipolysis. They concluded that dietary genistein may reduce the fattening process in ovariectomized rats. When Schleicher et al. (27) studied rats with accessory sex gland carcinoma, those treated with genistein had a decreased body weight compared with controls. Further animal studies will be required to verify that genistein promotes loss of body fat and to determine the optimal dose for such an effect.

Genistein holds great promise for nutrient-mediated regulation of body fat through its effects on adipocyte replication, differentiation, and lipolysis. Naringenin, on the other hand, failed to inhibit differentiation and may, therefore, be ineffective as an antiadipogenic compound.

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REFERENCES


