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 preand 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.

Genistein; naringenin; preadipocytes; adipocytes; peroxisome proliferator-activated receptor-γ; insulin receptor substrate-1

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 G0/G1 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 preconfluent preadipocytes. Although genistein inhibited MDI-induced proliferation of postconfluent preadipocytes and their subsequent differentiation into adipocytes, naringenin had no effect on these processes. In

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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-methyIxanthine were from Sigma (St. Louis, MO). Rabbit polyclonal anti-

signal 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 (PY20) 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 μM pyruvate at 37°C in 95% air–5% CO2. To induce differentiation, 3T3-L1 cells were seeded in 96-well plates at a density of 10,000 cells/100 μl well. Vehicle or 100 μM genistein or naringenin at the time of plating inhibited 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

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 postconfluent mitotic clonal expansion. After stimulation with MDI differentiation medium, growth-arrested postconfluent 3T3-L1 cells undergo two rounds of mitotic clonal expansion before exiting the cell cycle and expressing adipocyte-specific genes (18). We treated 2-day postconfluent preadipocytes with MDI plus 100 μM of flavonoids or vehicle and measured proliferation 72 h later. Although 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.

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...
would inhibit differentiation but that naringenin would not. We treated 2-day postconfluent preadipocytes with MDI plus 100 μM of 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 or vehicle. After 72 h, LDH was measured in supernatants and cell lysates. Cytotoxicity was expressed as % LDH release. Data shown reflect the means ± SE of 4 experiments. There was no significant difference between means.

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. Exposure of 3T3-L1 cells to genistein from days 3–5 after induction of differentiation did not alter adipogenesis. These findings demonstrate that genistein’s antidifferentiation 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 antidifferentiation 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-

Fig. 5. Effect of flavonoids on 3T3-L1 preadipocyte differentiation. Two-day postconfluent 3T3-L1 cells were differentiated according to the MDI protocol plus 100 μM of flavonoids or vehicle (day 0). On day 3, the differentiation medium was replaced with culture medium, which was changed every 2 days thereafter. On days 7–9 after induction of differentiation, cells were stained with Oil Red O (A), lysed for triglyceride and protein assays (B; data shown reflect the means ± SE of 3 experiments; different letters indicate statistically significant differences between means at P < 0.05), or lysed for immunoblot (IB) analysis (C; see MATERIALS AND METHODS). PPAR, peroxisome proliferator-activated receptor; STAT3, signal transducers and activators of transcription 3.
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) studied the effects of genistein on adipocyte lipolysis, and found that in isolated rat adipocytes, 1 mM of genistein enhances basal lipolysis but inhibits epinephrine-stimulated lipolysis.

Given that genistein promotes lipolysis and inhibits adipogenesis in cell culture, we anticipate that genistein will act similarly in vivo and potentially 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, elicits serum genistein to the 10–20 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.

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 of 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.

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