Genistein inhibits differentiation of primary human adipocytes☆

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Received 26 September 2007; received in revised form 14 December 2007; accepted 3 January 2008

Abstract

Genistein, a major soy isoflavone, has been reported to exhibit antiadipogenic and proapoptotic potential in vivo and in vitro. It is also a phytoestrogen which has high affinity to estrogen receptor γ. In this study, we determined the effect of genistein on adipogenesis and estrogen receptor (ER) α and β expression during differentiation in primary human preadipocytes. Genistein inhibited lipid accumulation in a dose-dependent manner at concentrations of 6.25 μM and higher, with 50 μM genistein inhibiting lipid accumulation almost completely. Low concentrations of genistein (3.25 μM) increased cell viability and higher concentrations (25 and 50 μM) decreased it by 16.48±1.35% (P<.0001) and 50.68±1.34% (P<.0001). Oil Red O staining was used to confirm the effects on lipid accumulation. The inhibition of lipid accumulation was associated with inhibition of glycerol-3-phosphate dehydrogenase activity and down-regulation of expression of adipocyte-specific genes, including peroxisome proliferator-activated receptor γ, CCAAT/enhancer binding protein α, glycerol-3-phosphate dehydrogenase, adipocyte fatty acid binding protein, fatty acid synthase, sterol regulatory element-binding protein 1, perilipin, leptin, lipoprotein lipase and hormone-sensitive lipase. These effects of genistein during the differentiation period were associated with down-regulation of ERα and ERβ expression. This study adds to the elucidation of the molecular pathways involved in the inhibition of adipogenesis by phytoestrogens.

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Keywords: Phytoestrogen; Adipogenesis; GPDH activity; Gene expression; Estrogen receptor

1. Introduction

Obesity is a risk factor for serious health problems associated with diabetes, coronary heart disease, hyperlipidemia and cancer, and its prevalence is rapidly rising [1–3]. As a result, there is increased urgency to develop strategies that will be effective for both the prevention and treatment of obesity. Fat mass can be regulated by various factors, including estrogens, which promote, maintain and control the distribution of body fat and alter adipose tissue metabolism. These steroids are known to regulate fat mass by increasing lipolysis and modulating the expression of genes that regulate lipid deposition in adipocytes [4]. This regulation mainly occurs through estrogen receptors (ER) α and β, which also mediate the action of several natural compounds, such as genistein.

Genistein (4,5,7-trihydroxyisoflavone), the most abundant isoflavone found in soybeans, has a heterocyclic diphenolic structure similar to estrogen [5]. It has been shown to decrease food intake, body weight and fat pad weight in ovariectomized female mice [6,7]. Genistein has

☆ This work was supported in part by grants from AptoTec, the Georgia Research Alliance and by the Georgia Research Alliance Eminent Scholar endowment held by C.A.B. and by a Korea Research Foundation Grant awarded to H.J. Park, funded by the Korean Government (KRF-2005-214-C00249).
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doi:10.1016/j.jnutbio.2008.01.006

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doi:10.1016/j.jnutbio.2008.01.006

2 αP2, adipocyte fatty acid binding protein; CEBPa, CCAAT/enhancer binding protein a; ER, estrogen receptor; FAS, fatty acid synthase; GPDH, Glyceral 3-phosphate dehydrogenase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PPARG, peroxisome proliferator-activated receptor g; SREBP, sterol regulatory element-binding protein.
been shown to inhibit lipid accumulation in 3T3-L1 cells [8–10] and also to inhibit cell proliferation and increase lipolysis in 3T3-L1 cells and rat adipocytes [8,11].

The process of adipogenesis, the development of mature fat cells from preadipocytes, includes alteration of cell shape, growth arrest and clonal expansion, followed by a complex sequence of changes in gene expression and storage of lipid [12]. This sequence of events is a result of the expression of adipocyte-specific genes such as PPARγ [13], C/EBPα [14] and adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element binding protein isoform [15].

Although genistein has been shown to have antiadipogenic and proapoptotic potential in vivo and in vitro, its effect on adipocyte specific gene expression and estrogen receptor expression in human adipocytes has not been studied. Therefore, we determined the effect of genistein on differentiation and on expression of adipocyte-specific genes and estrogen receptors in primary human maturing preadipocytes.

2. Methods and materials

2.1. Reagents

Genistein (99+%) was purchased from Indofine Chemical (Hillsborough, NJ, USA). AdipoRed Assay reagent was purchased from Cambrex BioScience (Walkersville, MA, USA) and CellTiter Blue Cell Viability Assay reagent was from Promega (Madison, WI, USA). Oil Red O stain and RNeasy Mini kit were from Sigma (St. Louis, MO, USA) and Qiagen (Valencia, CA, USA), respectively.

2.2. Cell cultures

The cells were purchased as cryopreserved preadipocytes from Zen Bio (Research Triangle Park, NC, USA). The cells originated from subcutaneous adipose tissue obtained from six females between 26 and 60 years of age with a body mass index 27.32 (range, 25.2–29.4) who were not diabetic and not smokers. The cells were cultured according to the manufacturer’s instructions with slight modification. Briefly, cryopreserved preadipocytes were passaged one time with preadipocyte medium (PM1; DMEM/Ham’s F-12 medium, HEPES, FBS, penicillin, streptomycin, amphotericin B; Zen-Bio) and then plated 40,625 cells/cm² with PM-1. Cells were fed every other day with PM-1 until confluent. To induce differentiation, PM-1 medium was replaced with differentiation medium (DM2; Zen-Bio) including bixin, pantothenate, human insulin, dexamethasone, isobutylmethylxanthine and a PPARγ agonist (Days 0–7). After 7 days, DM-2 medium was removed and cells were incubated for an additional 7 days with Adipocyte Medium (AM1; Zen-Bio; Days 7–14), which included PM-1, bixin, pantothenate, human insulin and dexamethasone. By Day 14, cells contained large lipid droplets and were considered mature adipocytes. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

2.3. Quantification of lipid content

Lipid content was quantified using commercially available AdipoRed Assay reagent according to the manufacturer’s instructions. AdipoRed, a solution of the hydrophilic stain Nile Red, is a reagent that enables the quantification of intracellular triglyceride. Briefly, cells were plated in 96-well plates, and genistein was added with DM-2 and AM-1 from Days 0–14. Medium with treatment was changed every 2–3 days. On Day 14, the treatment medium was removed and cells were rinsed with phosphate-buffered saline (PBS). Wells were then filled with 200 μl PBS, and 5 μl AdipoRed reagent was added. After incubation for 20 min at room temperature, fluorescent signal was measured with excitation at 485 nm and emission at 572 nm.

2.4. Cell viability assay

Maturing preadipocytes were incubated with genistein in 96-well plates during the adipogenesis period (Days 0–14) as described above. On Day 14, the treatment medium was removed and replaced with 100 μl fresh medium and 20 μl CellTiter Blue Cell Viability reagent (Promega). Cells were then incubated in dark for 1 h at 37°C and the fluorescent signal was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm to determine the resorufin concentration, which is proportional to the number of viable cells.

2.5. Oil red O staining

Cells were treated with genistein in six-well plates during the adipogenesis period (Days 0–14) as described above. On Day 14, cells were rinsed and scraped into 0.5 ml ice-cold sucrose buffer containing 0.28 M sucrose, 5 mM Tris, 1 mM EDTA and 0.002% β-mercaptoethanol and stored at −70°C. The homogenate was sonicated with three blasts for 15 s and centrifuged at 10,000 rpm, 10 min at 4°C. The supernatants were used for assay of GPDH activity according to Wise and Green [17]. Activities are expressed in mU/mg of protein (1 mU being equal to the oxidation of 1 nM of NADH/min). Protein was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.
2.7. Total RNA extraction

Primary human adipocytes were cultured in six-well plates and treated with genistein during the adipogenesis period (Days 0–14) as described above. On Days 0, 3, 7 and 14, RNA samples were extracted using the RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. RNA from undifferentiated preadipocytes was also extracted for comparing the expression levels of different genes. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.8. Real-time polymerase chain reaction

Five hundred nanograms of total RNA in a 20 μl reaction was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocols. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative polymerase chain reaction (PCR) (TaquinMan) assays were performed using 384-well Low-Density Array on the ABI PRISM 7900 Sequence Detection System. All of the oligonucleotide primer and fluorogenic probe sets for TaqMan real-time PCR (RT-PCR) were made by ABI (Table 1). The cycle conditions were: 94.5°C for 15 min, followed by 40 cycles of 97°C for 30 s and 59.7°C for 1 min. Expression of mRNAs was normalized by using 18S as an endogenous control to correct for differences in the amount of total RNA added to each reaction. The relative quantification values from each gene were used to compare the gene expression of control cells (the expression of genes on Day 0 for time course analysis and genes from cells treated with DMSO for analysis of significant treatment effects within individual time periods) to that of genistein-treated cells.

2.9. Statistical analysis

One-way analysis of variance (GLM procedure, Statistica, version 6.1; StatSoft) was used to determine significance of treatment effects. Fisher’s post hoc least significant difference test was used to determine significance of differences among means. Statistically significant differences are defined at the 95% confidence interval. Data shown are means±standard error.

3. Results

3.1. Genistein inhibited lipid accumulation

Primary human preadipocytes were treated with either 0.1% DMSO or genistein at various concentrations (3.125, 6.25, 12.5, 25 and 50 μM) during the differentiation period (Days 0–14), and lipid contents and cell viability were measured on Day 14. As shown in Fig. 1A, genistein inhibited lipid accumulation in a dose-dependent manner at concentrations of 6.25 μM and higher. The cells treated with 6.25, 12.5, 25 and 50 μM genistein decreased lipid accumulation by 34.36±0.97% (P<0.0001), 69.21±1.19% (P<0.0001), 89.70±1.40% (P<0.0001) and 94.58±0.65% (P<0.0001), respectively. Genistein treatment also affected cell viability (Fig. 1B). Cell viability was decreased by 25 and 50 μM genistein by 16.48±1.35% (P<0.0001) and 50.68±1.34% (P<0.0001), while 3.125 μM genistein increased viability by 7.68±5.30 (P<0.05). Viability was not affected by 6.25 and 12.5 μM genistein. Similar effects on lipid accumulation were observed using Oil Red O staining to visualize intracellular triglyceride in cells after treatment (Fig. 1C). The morphology of cells treated with 25 and 50 μM genistein was fibroblast-like.

3.2. Genistein decreased GPDH activity

GPDH activity was measured on Day 14 after treatment with genistein (Fig. 2). Genistein decreased GPDH activity in a dose-dependent manner. GPDH activity was decreased by 12.5, 25 and 50 μM genistein by 8.75±2.49% (P<0.05), 46.65±2.72% (P<0.001) and 85.55±1.03% (P<0.001), respectively. As expected, GPDH activity in undifferentiated preadipocytes was almost undetectable.

Table 1

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<th>Probe Sequence</th>
<th>Assay ID</th>
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3.3. Genistein down-regulated the expression of adipocyte-specific genes

To determine the time course responses of adipocyte-specific genes during the differentiation period, primary human preadipocytes were induced to differentiate under standard adipogenic conditions and total RNA was extracted on Days 3, 7 and 14 of the differentiation period. RT-PCR was performed to analyze the expression of the adipocyte specific genes PPARγ, aP2, C/EBPα, GPDH, FAS, SREBP1, perilipin, LPL and HSL.

The changes in gene expression in control cells over time are shown in Fig. 3. PPARγ was up-regulated during the differentiation period and peaked on Day 14, showing the greatest increase in expression between Days 3 and 7. The expression of FAS, SREBP1 and leptin also increased throughout the differentiation period, while the expression of aP2, C/EBPα, GPDH, perilipin, HSL and LPL increased up to Day 7.

To determine the effect of genistein on gene expression during the differentiation period, RNA from maturing preadipocytes treated with various concentrations of genistein (6.25, 25 and 50 μM) was extracted and RT-PCR was performed. Genistein treatment decreased the expression of all the above-mentioned genes. The mRNA levels of all these genes in undifferentiated preadipocytes were significantly lower than genistein-treated cells. The dose-dependent effect of genistein was most apparent on the peak expression days for each gene. This is shown for aP2, C/EBPα, GPDH, perilipin, HSL and LPL genes on Day 7.

Fig. 1. Lipid accumulation (A) and cell viability (B) in primary human preadipocytes treated with genistein during the differentiation period. The experiments were performed at least three times with eight replicates for each treatment. Oil Red O staining was also performed (C). Means that are not denoted with a common letter are different (P<.05).

Fig. 2. GPDH activity in primary human maturing preadipocytes treated with genistein during the differentiation period. The experiment was performed two times with three replicates per experiment for each treatment. Means that are not denoted with a common letter are different (P<.05).
3.4. Genistein altered the expression of estrogen receptors

After induction of differentiation, ERα expression was decreased up to 80.31±3.89% on Day 3 and then increased on Day 14 to 148.25±13.15% as compared to Day 0 (Fig. 5A). However, ERβ expression increased 3341.84±286.98% on Day 7 and then declined on Day 14 to 640.33±63.89% as compared to Day 0.

When cells were treated with genistein (Fig. 5B), the expression of ERα was decreased by 50 μM concentration on
Day 7 ($P<.05$) and by 25 μM on Day 14 ($P<.05$), whereas the expression of $ER\beta$ was decreased by all treatments by Day 7 ($P<.0005$) and by 6.25 and 25 μM on Day 14 ($P<.005$).

4. Discussion

Genistein has been shown to have potential anti-obesity effects, decreasing food intake, body weight and fat pad weight and inducing adipose tissue apoptosis in vivo [6,7] and inhibiting lipid accumulation and increasing lipolysis in vitro [8–11]. In this study, we examined the effect of genistein on differentiation, expression of adipocyte-specific genes and expression of $ER\alpha$ and $ER\beta$ in primary human preadipocyte culture. Genistein treatment during the differentiation period decreased lipid accumulation in a dose-dependent manner. This effect was observed with as little as 6.25 μM genistein, and 50 μM genistein inhibited it almost completely. This finding is consistent with those of other studies in murine adipocytes [7,10,18]. Genistein treatment with 25 μM and 50 μM also decreased cell viability during the differentiation period. The viability assay used in this study measures the metabolic capacity of cells as an indicator of cell viability, showing the total number of viable cells. Genistein has been reported to inhibit cell proliferation in 3T3-L1 adipocytes [8] and induce cell death in mice [6]. Consistently, total number of viable cells was decreased after high concentrations of genistein treatment in this study. It also indicates that the decreased lipid content with genistein treatment in the current study may be partially due to the decreased cell numbers resulting from inhibition of cell division and/or induction of cell death by genistein. We also demonstrated that the inhibition of lipid accumulation was associated with a decrease of GPDH activity, a marker of late adipocyte differentiation.

We next examined the expression of adipocyte specific genes in the same stage of cells to determine the potential mechanism of genistein’s antiadipogenic effect. Adipogenesis, the development of mature fat cells from preadipocytes, is an intensely studied model of cellular differentiation. Gregoire et al. [19] stated that the C/EBP family, PPAR family and SREBP1c showed early changes in gene expression during adipocyte differentiation, with maximal levels of $PPAR\gamma$ expression attained in mature adipocytes. $PPAR\gamma$ is the master adipogenic transcription factor and induces anabolic processes such as triacylglycerol synthesis by enhancing the transcription of genes encoding proteins such as $aP2$ [20] and LPL [21]. $PPAR\gamma$ and $C/EBP\alpha$ are thought to act synergistically to promote adipogenesis [22–24]. SREBP1c is also believed to potentiate adipogenesis, both by up-regulating $PPAR\gamma$ expression and by increasing availability of ligands for $PPAR\gamma$ through up-regulation of genes involved in lipid metabolism [25]. In contrast, during the terminal phase of differentiation, adipocytes in culture markedly increase de novo lipid synthesis and acquire sensitivity to insulin. The activity, protein and mRNA levels for enzymes involved in triacylglycerol metabolism including GPDH and FAS increase markedly. In addition, adipocytes synthesize other adipose tissue-specific products including $aP2$, perilipin and leptin [19]. LPL is secreted by mature adipocytes and plays a central role in controlling lipid accumulation. We measured the expression of $PPAR\gamma$, $aP2$, $C/EBP\alpha$, GPDH, FAS, SREBP1, perilipin, leptin, LPL and HSL genes in response to genistein treatment in the current study. First we determined the time course of expression of these genes during the differentiation period. Our findings varied somewhat from those of Gregoire et al. [19]. In our study, expression of $PPAR\gamma$, FAS, SREBP1 and leptin peaked at the end of the differentiation period, while expression of $aP2$, $C/EBP\alpha$, GPDH, perilipin, HSL and LPL peaked on Day 7. However, the study of Brown et al.
with primary human preadipocytes showed similar trends to our study, except for perilipin and HSL. When preadipocytes were treated with genistein during the differentiation period, expression of all the above adipocyte specific genes decreased. Consistently, Harmon et al. [8,9] found changes in PPARγ and C/EBPα expression, and Naaz et al. [7] found changes in LPL expression with inhibition of adipogenesis with genistein treatment in 3T3-L1 cells and mice, respectively.

Estrogens are known to play an important role in adipocyte development [4] and human adipose tissue expresses both the classic ERα and the recently discovered ERβ [27]. Furthermore, genistein can act as a phytoestrogen due to the presence of a phenolic ring necessary to bind ER and has shown a high affinity for ERβ [28]. Therefore, we determined whether genistein modified the expression of estrogen receptors during differentiation. First, we found that ERα and ERβ expression levels showed different patterns during differentiation. ERα expression decreased during the first 7 days and then increased to approximately 40% over predifferentiation levels by Day 14. In contrast, ERβ expression was almost undetectable prior to differentiation, then increased approximately 3300% by Day 7, followed by a decrease to about 640% higher than predifferentiation levels by Day 14. Joyner et al. demonstrated that human preadipocytes express the ERα but not the ERβ [29], and Crandall et al. indicated that human adipocytes expressed ERβ only after differentiation [30]. These reports are in agreement with our results that ERα was expressed in both pre- and mature adipocytes, but ERβ was expressed only after differentiation.

We also found that genistein treatment resulted in decreased expression of ERα and ERβ, with a greater effect on ERβ. This is consistent with the study of Penza et al. [18] who reported a greater effect on the down-regulation of ERβ as compared to ERα after both acute and chronic genistein treatment in mice. Genistein has also been reported to have approximately a 30-fold-higher affinity to ERβ than to ERα [28], but in a cell-based gene transcription assay, genistein was only four- to five-fold more potent in ERβ- than ERα-linked transcription [31]. The effect of ER on adipogenesis has not been thoroughly investigated. However, Naaz et al. [32] showed that ERβ has an adipogenic role in adipose tissue. Therefore, the antiadipogenic effect of genistein in our study might be partially mediated through the down-regulation of ERβ expression.

The range of concentrations used in the current study is comparable to the serum concentrations achieved in animal or human studies. These values are higher than those reported in Japanese men consuming a low-fat diet with high content of soy products [33] or in postmenopausal women after oral administration of genistein (54 mg/day) [34]. However, other
studies reported that serum genistein concentrations can reach up to 6.6 μM for soy-based formula-fed infants [35] and 2.4 μM for adults ingesting soybean powder [36]. In mice, a dose of approximately 150 mg/kg per day genistein administered to ovariectomized female mice resulted in a serum genistein concentration of 3.8 μM [7]. This same dose was shown to cause weight loss [6,7] and adipose tissue apoptosis [6], whereas 100 μM genistein was the minimum concentration required to demonstrate a significant increase in apoptosis of 3T3-L1 adipocytes in vitro after a 24-h incubation period [6]. These studies indicate the difficulty in making predictions about relationships between concentrations shown effective in vitro under somewhat artificial conditions and effective serum levels of the same agent. However, the recent popularity of soy supplements makes it possible to consume amounts several-fold greater than those obtained even with a high soy diet. Thus, we assume that the genistein concentrations in our study may be in a range achieved in populations consuming high amounts of soy-containing products.

In conclusion, we showed that genistein inhibited adipogenesis through down-regulating adipocyte specific transcription factors. Furthermore, genistein down-regulated both ERα and ERβ during the differentiation process in primary human preadipocytes. Although antiadipogenic effects of genistein have been previously investigated, this is the first study to report the effect of genistein on inhibiting adipogenesis in primary human adipocytes. Further, this is the first study to investigate the effect of genistein on human primary adipocyte gene expression throughout the differentiation process. This study adds to the elucidation of the molecular pathways of phytoestrogens that have specific effects on adipocytes.

References


