Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 140–148
Journal of Nutritional Biochemistry

Genistein inhibits differentiation of primary human adipocytes☆

Hea Jin Park[a], Mary Anne Della-Fera[a,1], Dorothy B. Hausman[b], Srujana Rayalam[a], Suresh Ambati[a], Clifton A. Baile[a,b,*,1]

[a]Department of Animal & Dairy Science, University of Georgia, Athens, GA 30602-2771, USA
[b]Department of Foods and Nutrition, University of Georgia, Athens, GA 30602-2771, USA

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 β. 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.

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[2], 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).

* Corresponding author. 444 Edgar L. Rhodes Center for Animal and Dairy Science, University of Georgia, Athens, GA 30602-2771, USA. Tel.: +1 706 542 2771; fax: +1 706 542 7925.

E-mail address: cbaile@uga.edu (C.A. Baile).

[1] Drs. Baile and Della-Fera are investors in and serve on the Board of Directors for AptoTec.

[2] *aP2*, adipocyte fatty acid binding protein; *C/EBPα*, CCAAT/enhancer binding protein a; *ER*, estrogen receptor; *FAS*, fatty acid synthase; *GPDH*, Glycerol 3-phosphate dehydrogenase; *HSL*, hormone-sensitive lipase; *LPL*, lipoprotein lipase; *PPARγ*, peroxisome proliferator-activated receptor g; *SREBP*, sterol regulatory element-binding protein.

0955-2863/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.01.006

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/cm^2 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 biotin, 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, biotin, 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% CO_2 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 washed with PBS and fixed with Baker's formalin (10 ml 37% formaldehyde, 10 ml of a 10% calcium chloride solution, 80 ml distilled water) for 30 min at room temperature and then stained with Oil Red O and hematoxylin [16]. After staining, cells were mounted with glycerol gelatin and the images of each dish were captured using ImagePro software (MediaCybernetics, Silver Spring, MD, USA).

2.6. Glycerol 3-phosphate dehydrogenase activity

In addition to Oil Red O staining, *GPDH* activity was used as a marker of late adipocyte differentiation. Primary human preadipocytes were cultured in six-well plates and treated with genistein during the adipogenesis period (Days 0–14) as mentioned 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) (TaqMan) 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<.0001), 69.21±1.19% (P<.0001), 89.70±1.40% (P<.0001) and 94.58±0.65% (P<.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<.0001) and 50.68±1.34% (P<.0001), while 3.125 μM genistein increased viability by 7.68±5.30 (P<.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<.05), 46.65±2.72% (P<.001) and 85.55±1.03% (P<.001), respectively. As expected, *GPDH* activity in undifferentiated preadipocytes was almost undetectable.

Table 1
List of probes for genes used in real time RT-PCR

Gene Symbol	Gene Name	Probe Sequence	Assay ID
18S	Eukaryotic 18S rRNA	CCATTGGAGGGCAAGTCTGGTGCCA	Hs99999901_s1
PPARγ	Peroxisome proliferator-activated receptor γ	TCTCAGTGGAGACCGCCCAGGTTTG	Hs00234592_m1
aP2	Adipocyte fatty acid binding protein	TATGAAAGAAGTAGGAGTGGGCTTT	Hs00609791_m1
C/EBPα	CCAAT/enhancer binding protein α	CAAATCGTGCCTTGTCATTTTATTT	Hs00269972_s1
GPDH	Glycerol-3-phosphate dehydrogenase 1	CCCCCAAATGTGGTGGCTGTCCCAG	Hs00193386_m1
FAS	Fatty acid synthase	CCCTGGCCAAGGTGCTGCTGTCCCT	Hs00188012_m1
SREBP1	Sterol regulatory element-binding protein 1	GTGGGCACTGAGGCAAAGCTGAATA	Hs01088691_m1
perilipin	Perilipin	GAGACCTCCCTGAGCAGGAGAATGT	Hs00160173_m1
leptin	Leptin	GACATTTCACACACGCAGTCAGTCT	Hs00174877_m1
LPL	Lipoprotein lipase	ATGCCCTACAAAGTCTTCCATTACC	Hs00173425_m1
HSL	Hormone-sensitive lipase	AGGATACAGCCTCAAGGCTCATCCA	Hs00193510_m1
ERα	Estrogen receptor α	GATGAAAGGTGGGATACGAAAAGAC	Hs00174860_m1
ERβ	Estrogen receptor β	TGGTGAAGTGTGGCTCCCGGAGAGA	Hs00230957_m1

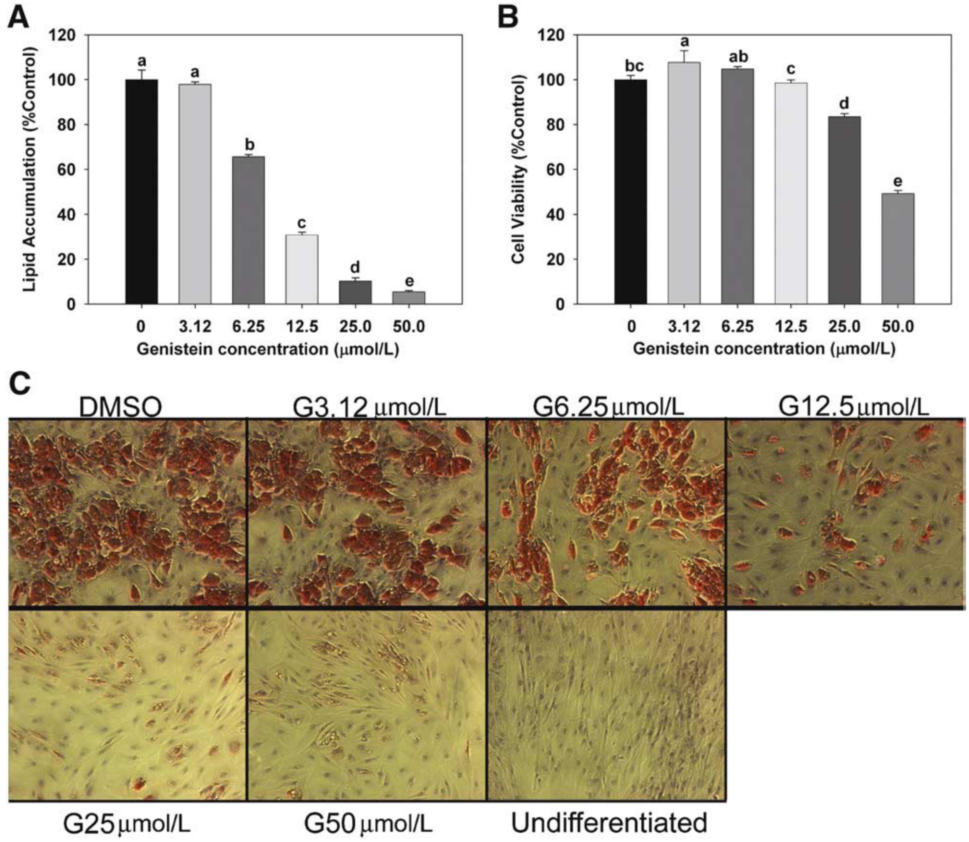

Fig. 1. Lipid accumulation (A) and cell viability (B) in primary human preadipocytes treated with genistein during the differentiation period. The experiments were performed at least three times with eight replicates for each treatment in each experiment. Oil Red O staining was also performed (C). Means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

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*.

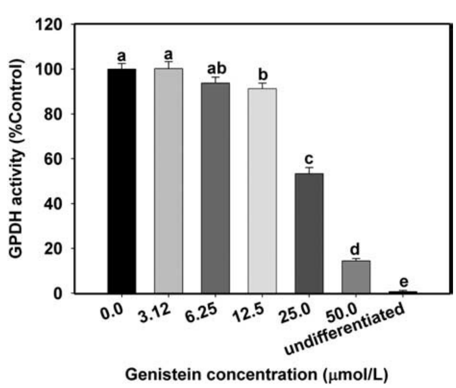

Fig. 2. *GPDH* activity in primary human maturing preadipocytes treated with genistein during the differentiation period. The experiment was performed two times with three replicates per experiment for each treatment. Means that are not denoted with a common letter are different (P<.05).

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

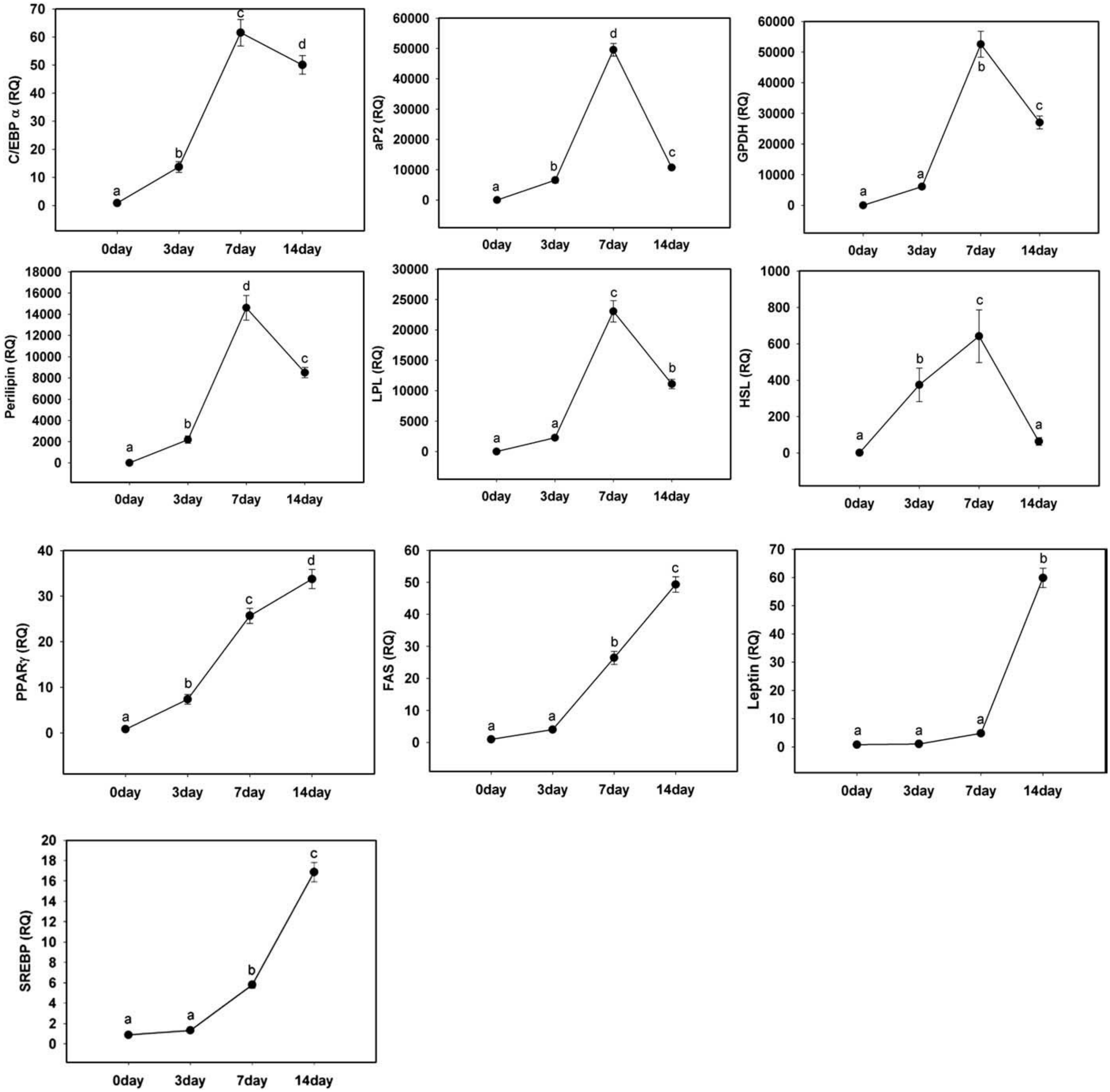

Fig. 3. Time course of changes in gene expression in untreated maturing preadipocytes. Means that are not denoted with a common letter are different (P<.05). The experiments were performed in four to six replicates for each treatment.

(Fig. 4A) and for *PPARγ*, *FAS*, *SREBP1* and *leptin* genes on Day 14 (Fig. 4B). On Day 7, 25 and 50 μM genistein decreased the expression of *C/EBPα* (P<.001), *GPDH* (P<.0005) and *perilipin* (P<.001), while expression of *aP2* (P<.005), *HSL* (P<.05) and *LPL* (P<.0005) were decreased with concentrations of 6.25 μM genistein and higher. On Day 14, 25 and 50 μM genistein decreased the expression of *PPARγ* (P<.0001), *FAS* (P<.0001) and *SREBP1* (P<.0001), while *leptin* expression was decreased with concentrations of 6.25 μM genistein and higher (P<.0001).

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

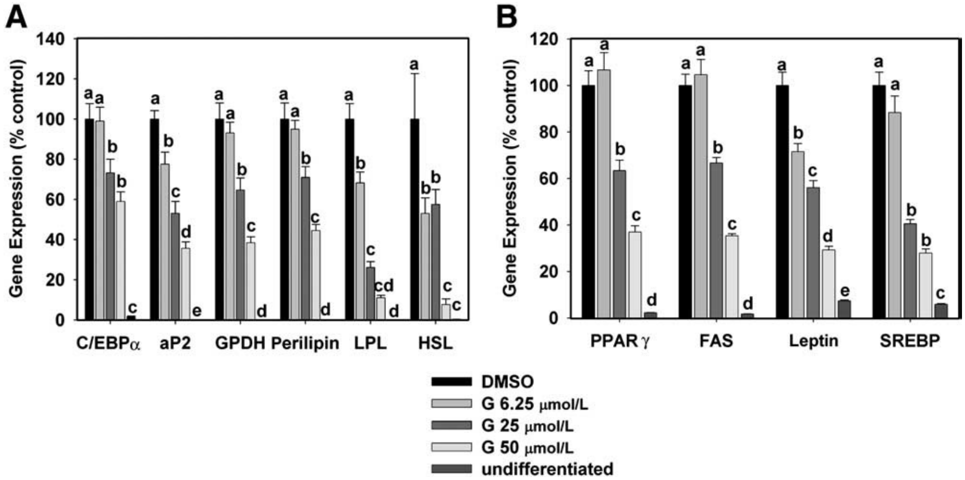

Fig. 4. (A) Genistein-induced alterations of mature adipocyte gene expression after 7 days incubation. (B) Genistein-induced alterations of mature adipocyte gene expression after 14 days incubation. For each gene, means that are not denoted with a common letter are different (P<.05). The experiments were performed in four to six replicates for each treatment.

Day 7 (P<.05) and by 25 μM on Day 14 (P<.05), whereas the expression of *ERβ* 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 *ERα* and *ERβ* 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 *SREBP1*c showed early changes in gene expression during adipocyte differentiation, with maximal levels of *PPARγ* expression attained in mature adipocytes. *PPARγ* 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]. *PPARγ* and *C/EBPα* are thought to act synergistically to promote adipogenesis [22–24]. *SREBP1*c is also believed to potentiate adipogenesis, both by up-regulating *PPARγ* expression and by increasing availability of ligands for *PPARγ* 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 *PPARγ*, *aP2*, *C/EBPα*, *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 *PPARγ*, *FAS*, *SREBP1* and *leptin* peaked at the end of the differentiation period, while expression of *aP2*, *C/EBPα*, *GPDH*, *perilipin*, *HSL* and *LPL* peaked on Day 7. However, the study of Brown et al.

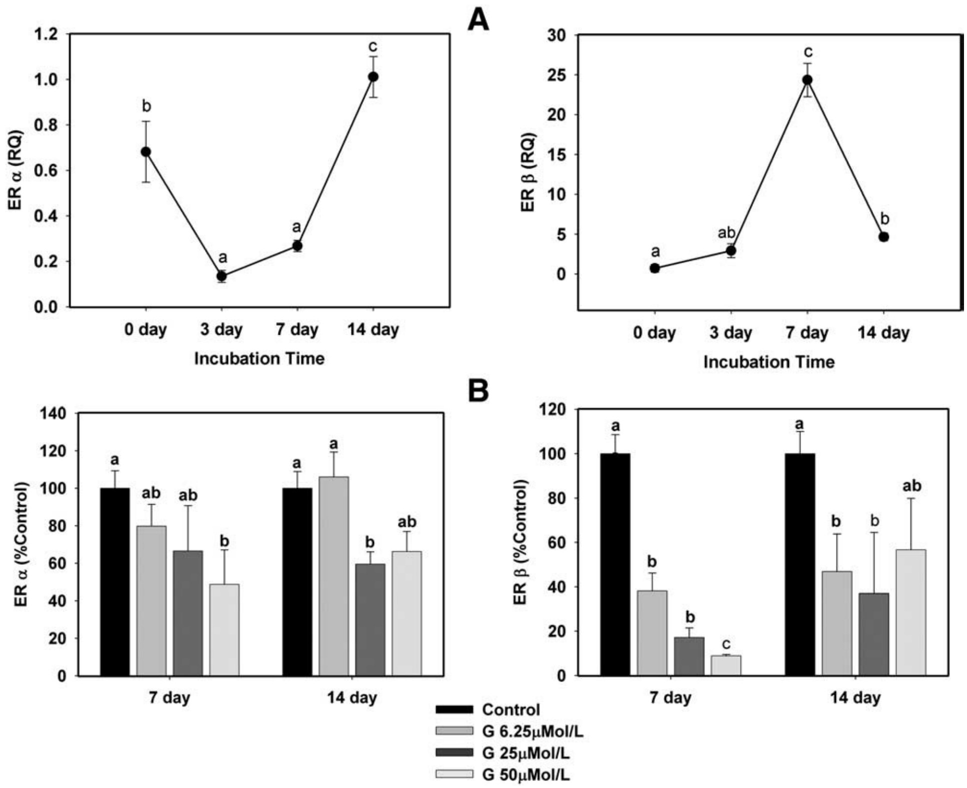

Fig. 5. (A) Time course of estrogen receptor gene expression in untreated maturing preadipocytes. Means not denoted with a common letter are different ($P<.05$). (B) Effect of genistein treatment during the differentiated period on estrogen receptor gene expression. The experiments were performed in four to six replicates for each treatment. Within the same time period, means that are not denoted with a common letter are different ($P<.05$).

[26] 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

[1] Lazar MA. How obesity causes diabetes: not a tall tale. Science 2005;307:373–5.

[2] Zimmermann-Belsing T, Feldt-Rasmussen U. Obesity: the new worldwide epidemic threat to general health and our complete lack of effective treatment. Endocrinology 2004;145:1501–2.

[3] Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999-2004. JAMA 2006;295:1549–55.

[4] Cooke PS, Naaz A. Role of estrogens in adipocyte development and function. Exp Biol Med (Maywood) 2004;229:1127–35.

[5] Adlercreutz CH, Goldin BR, Gorbach SL, Hockerstedt KA, Watanabe S, Hamalainen EK, et al. Soybean phytoestrogen intake and cancer risk. J Nutr 1995;125:757S–70S.

[6] Kim HK, Nelson-Dooley C, Della-Fera MA, Yang JY, Zhang W, Duan J, et al. Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. J Nutr 2006;136:409–14.

[7] Naaz A, Yellayi S, Zakroczymski MA, Bunick D, Doerge DR, Lubahn DB, et al. The soy isoflavone genistein decreases adipose deposition in mice. Endocrinology 2003;144:3315–20.

[8] Harmon AW, Harp JB. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. Am J Physiol Cell Physiol 2001;280: C807–13.

[9] Harmon AW, Patel YM, Harp JB. Genistein inhibits CCAAT/enhancer-binding protein beta (C/EBPbeta) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression. Biochem J 2002;367:203–8.

[10] Hwang JT, Park IJ, Shin JI, Lee YK, Lee SK, Baik HW, et al. Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. Biochem Biophys Res Commun 2005;338:694–9.

[11] Kandulska K, Nogowski L, Szkudelski T. Effect of some phytoestrogens on metabolism of rat adipocytes. Reprod Nutr Dev 1999;39:497–501.

[12] Gregoire FM. Adipocyte differentiation: from fibroblast to endocrine cell. Exp Biol Med (Maywood) 2001;226:997–1002.

[13] Zhu Y, Qi C, Korenberg JR, Chen XN, Noya D, Rao MS, et al. Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms. Proc Natl Acad Sci U S A 1995;92:7921–5.

[14] Cao Z, Umek RM, McKnight SL. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 1991;5:1538–52.

[15] Kim JB, Spiegelman BM. ADD1/*SREBP1* promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev 1996;10:1096–107.

[16] Suryawan A, Hu CY. Effect of serum on differentiation of porcine adipose stromal-vascular cells in primary culture. Comp Biochem Physiol Comp Physiol 1993;105:485–92.

[17] Wise LS, Green H. Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. J Biol Chem 1979;254:273–5.

[18] Penza M, Montani C, Romani A, Vignolini P, Pampaloni B, Tanini A, et al. Genistein affects adipose tissue deposition in a dose-dependent and gender-specific manner. Endocrinology 2006;147:5740–51.

[19] Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol Rev 1998;78:783–809.

[20] Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 1994;8:1224–34.

[21] Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, et al. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. Embo J 1996;15:5336–48.

[22] Tang QQ, Lane MD. Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes Dev 1999;13:2231–41.

[23] Cornelius P, MacDougald OA, Lane MD. Regulation of adipocyte development. Annu Rev Nutr 1994;14:99–129.

[24] Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, et al. *C/EBPa*lpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 2002;16:22–6.

[25] Kim JB, Wright HM, Wright M, Spiegelman BM. ADD1/*SREBP1* activates PPARgamma through the production of endogenous ligand. Proc Natl Acad Sci U S A 1998;95:4333–7.

[26] Brown JM, Boysen MS, Jensen SS, Morrison RF, Storkson J, Lea-Currie R, et al. Isomer-specific regulation of metabolism and PPARgamma signaling by CLA in human preadipocytes. J Lipid Res 2003;44:1287–300.

[27] Pedersen SB, Bruun JM, Hube F, Kristensen K, Hauner H, Richelsen B. Demonstration of estrogen receptor subtypes alpha and beta in human adipose tissue: influences of adipose cell differentiation and fat depot localization. Mol Cell Endocrinol 2001;182:27–37.

[28] Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252–63.

[29] Joyner JM, Hutley LJ, Cameron DP. Estrogen receptors in human preadipocytes. Endocrine 2001;15:225–30.

[30] Crandall DL, Busler DE, Novak TJ, Weber RV, Kral JG. Identification of estrogen receptor beta RNA in human breast and abdominal

subcutaneous adipose tissue. Biochem Biophys Res Commun 1998;248:523–6.

[31] Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol 1998;54:105–12.

[32] Naaz A, Zakroczymski M, Heine P, Taylor J, Saunders P, Lubahn D, et al. Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). Horm Metab Res 2002;34:758–63.

[33] Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 1993;342:1209–10.

[34] Atteritano M, Marini H, Minutoli L, Polito F, Bitto A, Altavilla D, et al. Effects of the phytoestrogen genistein on some predictors of cardiovascular risk in osteopenic, postmenopausal women: a two-year randomized, double-blind, placebo-controlled study. J Clin Endocrinol Metab 2007;92:3068–75.

[35] Setchell KD, Zimmer-Nechemias L, Cai J, Heubi JE. Exposure of infants to phyto-oestrogens from soy-based infant formula. Lancet 1997;350:23–7.

[36] Watanabe S, Yamaguchi M, Sobue T, Takahashi T, Miura T, Arai Y, et al. Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). J Nutr 1998;128:1710–5.