Genistein Suppresses Adipogenesis of 3T3-L1 Cells via Multiple Signal Pathways

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Genistein, an isoflavone, was shown to have therapeutic effects for obesity, diabetes and cardiovascular diseases. This study investigated the effect and underlying mechanism of genistein on adipogenesis in 3T3-L1 preadipocytes. Genistein increased lipid accumulation and decreased the nonesterified fatty acid (NEFA) content of 3T3-L1 on day 6 after the induction of differentiation with methylisobutylxanthine, dexamethasone and insulin (MDI). Genistein recovered nitric oxide (NO) release suppressed by MDI and the results were consistent with the expression of endothelial NO synthase (eNOS) assayed by western blotting. Pretreatment with genistein inhibited the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK, p38) and janus kinase/signal transducers and activators of the transcription (JAK/STAT) cascade.

NO is an important mediator with a wide variety of biological functions. Dietary supplementation with arginine promoted lipolysis and fatty acid oxidation in adipose tissue of adult ZDF rats (Fu et al., 2005). An NO donor, S-nitroso-N-acetylpenicillamine (SNAP), affects basal or stimulated lipolysis in adipocytes (Gaudiot et al., 1998; Fruhbeck and Gomez-Ambrosi, 2001). p38, a stress-activated MAPK, is capable of transducing a multitude of signals for development and homeostasis in animals (Rawlings et al., 2004). The expression of STATs 1, 5A and 5B is highly induced during differentiation and correlates with lipid accumulation in 3T3-L1 cells (Stephens et al., 1996).

Genistein, a soy-derived isoflavone, has been found to have therapeutic effects for obesity, diabetes and cardiovascular diseases. Recently, some studies reported that genistein blocks adipogenesis and increases lipolysis in 3T3-L1 adipocytes (Harmon et al., 2002; Harmon and Harp, 2001; Hwang et al., 2005) and rat adipocytes (Kandulska et al., 1999; Skudelska et al., 2000). Genistein produced a suppression effect on adipocyte differentiation by inhibiting the tyrosine phosphorylation of CCAAT/enhancer binding protein beta (C/EBPβ) (Harmon et al., 2002), inducing the expression of peroxisome proliferator-activated receptor alpha (PPARα) (Kim et al., 2004) and activating AMP-activated protein kinase (Hwang et al., 2005). Although these reports showed an inhibitory effect of genistein on adipogenesis, several aspects of the underlying mechanism are unclear.

The present study investigated the role of NO, p38 and JAK2 pathway in genistein-inhibited adipocyte differentiation.

INTRODUCTION

The induction of obesity is mainly dependent on the regulation of adipocyte differentiation (Shimomura et al., 1993), and involves multiple signal pathways; for example, nitric oxide (NO), p38 mitogen-activated protein kinase (p38 MAPK, p38) and janus kinase/signal transducers and activators of the transcription (JAK/STAT) cascade.

MATERIAL AND METHODS

Materials. Mouse anti-fatty acid synthase (FAS) antibody was from Transduction Laboratories (San Diego, USA), rabbit anti-endothelial NO synthase (eNOS) antibody was from Calbiochem (San Diego, USA), and goat anti-human C/EBPβ antibody was from Transduction Laboratories (San Diego, USA). Materials were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.
rabbit anti-phospho-p38, anti-C/EBPα antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, USA), rabbit anti-phospho-JAK2 antibodies were from Upstate Biotechnology (Lake Placid, USA) and mouse anti-α-tubulin antibody was from Molecular Probes (Eugene, USA). Genistein, insulin and AG490 were from Wako Pure Chemicals (Osaka, Japan), methylisobutyxanthine (MIX), dexamethasone were from ICN Biomedicals Inc. (Costa Mesa, USA), SB203580 was from EMD Biosciences Inc. (San Diego, USA). The nonesterified fatty acid (NEFA) assay kit was from Wako Pure Chemicals (Osaka, Japan). The NO assay kit was from Dojindo (Kumamoto, Japan). The other reagents used were of the highest grade commercially available.

**Cell culture.** 3T3-L1 preadipocytes (Human Science Cell Research Resources, Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 95% air, 5% CO2. At 2 days postconfluent the 3T3-L1 preadipocytes (day 0) were stimulated with 0.5 mM MIX, 0.5 μm dexamethasone and 10 μg/mL insulin (MDI) added to DMEM/0.3% FBS culture medium to induce differentiation, which was changed every 2 days thereafter until analysis. Genistein was used at 50 μM as the end concentration.

**Detection of NEFA.** The NEFA content in the conditioned medium was measured with an enzymatic colorimetric assay kit (Wako, Osaka, Japan) according to the manufacturer’s protocol. The sample was mixed with reagent 1 and incubated for 10 min at 37 °C. Then, reagent 2 was added and after 10 min incubation at 37 °C, a colored product was formed with a maximal absorbance at 550 nm. The data were calibrated using the standard curve.

**NO measurement.** The NO level in the conditioned medium was measured colorimetrically using a NO assay kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. The sample was mixed with enzyme for 2 h at room temperature. Reagent A was added and incubated for 5 min, then reagent B was added. After incubation for 10 min, the NO level was detected spectrophotometrically at 560 nm and the data were calibrated with the standard curve.

**Western blotting.** The 3T3-L1 adipocytes in 100 mm dishes were stimulated with MDI and genistein for the indicated time, and whole cell extracts were prepared by lysing the cells in extraction buffer (RIPA buffer) containing 50 mMol/L Tris(hydroxymethyl)aminomethane (Tris)/HCI, pH 8.0, 150 mMol/L NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mMol/L dithiothreitol (DTT), 0.05 mMol/L phenylmethyl-sulfonyl-fluoride (PMSF), 0.002 mg/mL aprotinin, 0.002 mg/mL leupeptin and 1 mMol/L NaVO3 after stimulation. The protein concentration was quantified with Bio-Rad DC protein assay reagent (Bio-Rad, Hercules, USA). Equal amounts of protein were mixed with SDS sample buffer and incubated for 5 min at 100 °C before loading. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Amersham Biosciences. Immunoreactive bands were detected by means of an ECL plus western blotting detection system (Amersham Biosciences, Little Chalfont, UK). The chemiluminescent signals were scanned from films (Nippon Polaroid K.K., Tokyo, Japan), and imported into Adobe Photoshop (Adobe, San Jose, USA). Quantitative analysis was performed by NIH ImageJ 1.36b software.

**Results.** The results are expressed as the mean ± SEM. One-way ANOVA was performed to assess differences in parameters between groups using the LSD test. A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Genistein inhibited MDI-induced lipid accumulation and the production of NEFA in 3T3-L1 cells (Fig. 1).** Stimulation with MDI increased the production of NEFA from 16.96 ± 0.83 μEq/L/μg protein in the control to 22.31 ± 1.04 μEq/L/μg protein, and the level decreased to 17.01 ± 0.58 μEq/L/μg protein with genistein treatment (n = 6, p < 0.01 vs MDI).

**Genistein recovered the suppressed eNOS expression and NO production in 3T3-L1 cells.** The NO production decreased significantly after induction by MDI (1.18 ± 0.05 μmol/L/μg protein), whereas it recovered to 1.65 ± 0.13 μmol/L/μg protein with genistein treatment (Fig. 2A) (n = 6, p < 0.01 vs MDI). This result was consistent with the effect of genistein on eNOS protein expression assayed by western blotting (Fig. 2B). The expression changes in each sample are shown relative to the control value (100%). MDI inhibited eNOS expression to 62% on day 6 after the induction of differentiation and genistein recovered it to 93% (n = 3, p < 0.01 vs MDI).

**Genistein inhibited insulin-induced FAS expression via the p38 signal pathway in 3T3-L1 cells.** p38 was rapidly phosphorylated following stimulation with 10 μg/mL of insulin and the maximal increase in the tyrosine phosphorylation of p38 in response to insulin was 183% at 5 min (Fig. 3A). However, densitometric analysis showed

![Figure 1](https://example.com/f1.png)
Figure 2. Effect of genistein on NO production and eNOS expression in 3T3-L1 cells. (A) Two-day postconfluent 3T3-L1 cells (day 0) were induced to differentiate in the presence of vehicle or 50 μM genistein. Medium was collected and assayed for the content of NO on day 6 after induction of differentiation. Data are expressed as the mean ± SEM (n = 6). ** p < 0.01; *** p < 0.001. (B) Two-day postconfluent 3T3-L1 cells (day 0) were induced to differentiate in the presence of vehicle, 50 μM genistein. eNOS expression was analysed by western blotting on day 6 after induction of differentiation. The data in each panel are representative of three independent experiments. Each of the three separate experiments yielded similar results. Data are expressed as the mean ± SEM (n = 3). ** p < 0.01; *** p < 0.001.

Figure 3. Effect of genistein on the expression of phospho-p38 and FAS. (A) 3T3-L1 cells were growth-arrested in serum-free DMEM for 16–24 h and then treated with different condition media (serum-free DMEM, 10 μg/mL insulin and 10 μg/mL insulin plus 50 μM genistein, respectively) for the indicated times following either no pretreatment (insulin group) or pretreatment with genistein (insulin plus genistein group) for 30 min as shown in lanes 1–6. The phosphorylation of p38 was analysed by western blotting using specific anti-phospho-p38 antibody. (B and C) Two-day postconfluent 3T3-L1 cells (day 0) were induced to differentiate in the presence of vehicle, 50 μM genistein or 5 μM SB203580. FAS expressions were analysed by western blotting using specific anti-FAS antibody on day 6 after induction of differentiation. Data in each panel are representative of three independent experiments. Each of the three separate experiments yielded similar results. Data are expressed as the mean ± SEM (n = 3). * p < 0.05; ** p < 0.01; *** p < 0.001.
that p38 phosphorylation was significantly inhibited by simultaneous pretreatment with 50 μM of genistein for 30 min at 5 min (94%) \((n = 3, \ p < 0.01 \ \text{vs MDI})\).

The study also tested the effect of genistein on FAS expression (Fig. 3B). The expression of FAS increased significantly (178%) compared with the control on day 6 after stimulation of MDI; however, the expression of FAS decreased to 74% with genistein treatment \((n = 3, \ p < 0.001 \ \text{vs MDI})\). Moreover, the FAS expression of 3T3-L1 was associated with the activation of the p38 pathway (Fig. 3C). SB203580, a specific inhibitor of p38, mimicked the FAS inhibition effect of genistein and decreased FAS expression from 334% with MDI stimulation to 56% \((n = 3, \ p < 0.01 \ \text{vs MDI})\).

**Genistein inhibited JAK2-mediated adipocyte differentiation in 3T3-L1 cells.** JAK2 was rapidly phosphorylated within 15 min and peaked at 60 min in 3T3-L1 following stimulation with MDI (Fig. 4A). Densitometric analysis showed that the phosphorylation of JAK2 in response to MDI was 174% at 5 min, 227% at 60 min and 200% at 180 min, and was significantly inhibited to 55%, 39% and 50%, respectively, by pretreatment with genistein for 30 min \((n = 3, \ p < 0.01 \ \text{vs MDI})\).

The study tested the effect of genistein or AG490, an inhibitor of JAK2, on C/EBPα expression (Fig. 4B) in order to investigate whether genistein inhibited adipocyte differentiation via JAK2-mediated C/EBPα expression. The expression of C/EBPα increased significantly compared with the control on day 6 after stimulation with MDI; however, the expression of C/EBPα was decreased with genistein or AG490 treatment.

**DISCUSSION**

Adipocyte differentiation plays an important role during the induction of obesity, which increases the risk for cardiovascular diseases and type-2 diabetes (Shimonmura et al., 1998). Genistein, an isoflavone derived from soybean products, was shown to have therapeutic effects on obesity (Cooke and Naaz, 2005). The data also show that on day 6 after treatment with MDI, genistein inhibited lipid accumulation and decreased significantly the NEFA content in 3T3-L1 preadipocytes (Fig. 1). The results indicate that genistein administration may be effective therapy for adipocyte differentiation.

In the present study, stimulation with MDI inhibited the content of NO and the expression of eNOS in 3T3-L1 cells (Fig. 2). Previous studies showed that dexamethasone, one of the components of MDI, decreased the NO level and NOS expression both in vivo and in vitro (Rees et al., 1990; Dudek et al., 1994). Treatment with genistein recovered the decreased NO and eNOS to a higher level (Fig. 2). Gaudiot et al. demonstrated that SNAP, a NO donor, had effects on basal or stimulated lipolysis in isolated adipocytes (Gaudiot et al., 1998, 2000). The results indicate that genistein stimulated lipolysis, at least in part, by preventing the inhibiting effect of dexamethasone on eNOS expression and NO release.

While p38 is present throughout adipocyte differentiation, p38 kinase activity and phosphorylation are observed only during the earliest phases of differentiation (Engelman et al., 1999). The results that p38 was rapidly phosphorylated and peaked at 5 min after stimulation with insulin are consistent with this study (Fig. 3A). p38 activation is required for adipocyte differentiation in 3T3-L1 (Takenouchi et al., 2004). Engelman et al. (1998, 1999) showed that the addition of p38 inhibitors early in 3T3-L1 differentiation decreased adipocyte formation. In the present study, pretreatment with genistein inhibited the high level of p38 phosphorylation, which demonstrated that genistein suppressed adipocyte differentiation partially by suppressing the phosphorylation of p38 (Fig. 3A).

Insulin plays a major role in the modulation of key genes in lipid metabolism and triglyceride storage including FAS (Claycombe et al., 1998). The administration of insulin to streptozotocin-diabetic mice stimulated the level of FAS mRNA and FAS transcription rates (Paulauskis and Sul, 1989). The findings that stimulation with MDI increased the expression of FAS in 3T3-L1 preadipocytes are in agreement with the previous studies (Fig. 3B). FAS plays a central role in de novo lipogenesis in mammals and birds (Wakil...
et al., 1983). FAS mRNA levels increased dramatically during 3T3-L1 adipocyte differentiation (Paulauskis and Sul, 1988; Moustaid and Sul, 1991). Loftus et al. (2000) showed that the inhibition of fatty acid synthase reduced food intake, and caused weight loss. In this study, increased expression of FAS induced by MDI was significantly inhibited by genistein treatment (Fig. 3B), implying that genistein-blocked adipocyte differentiation in 3T3-L1 preadipocyte was related with the down-regulation of FAS expression.

It was also found that SB203580, a specific inhibitor of p38, mimicked the FAS inhibition effect of genistein (Fig. 3C), which indicated that the FAS expression of 3T3-L1 may be associated with activation of the p38 pathway, and the effect of genistein was partially due to the inhibition of p38 and then FAS.

In the present study, phosphorylation of JAK2 induced by MDI was prevented by genistein pretreatment (Fig. 4A). JAK activation stimulates cell proliferation, differentiation, migration and apoptosis. These cellular events are critical to hematopoiesis, immune development, adipogenesis and other processes (Rawlings et al., 2004). Activation of JAK2 mediates growth hormone (GH) effects in preadipocytes, as the depletion of JAK2 severely attenuates the ability of GH to promote adipocyte differentiation of 3T3-F442A preadipocytes (Yarwood et al., 1999). Furthermore, genistein or AG490, an inhibitor of JAK2, inhibited the expression of CEBPα, a marker of adipocyte differentiation (Fig. 4B). CEBPα, one of the members of the bZIP family, is an important transcription factor for adipogenesis. CEBPα is expressed at high levels in terminally differentiated cells of adipose tissue. The expression of antisense C/EBPα RNA in 3T3-L1 cells blocks differentiation (Lin and Lane, 1992). The results suggest that JAK2-mediated CEBPα expression was involved in the suppression effect of genistein on adipogenesis.

In summary, genistein inhibited 3T3-L1 adipocyte differentiation by improving eNOS expression, inhibiting p38 phosphorylation and decreasing FAS expression, and preventing JAK2-mediated CEBPα expression. The data suggest that genistein may have potential for the regulation of body fat through its effects on preadipocyte differentiation and lipolysis.

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REFERENCES


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