Genistein inhibits CCAAT/enhancer-binding protein β (C/EBP β) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression

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The tyrosine kinase inhibitor genistein inhibits 3T3-L1 adipogenesis when present during the first 72 h of differentiation. In this report, we investigated the underlying mechanisms involved in the anti-adipogenic effects of genistein. We found that genistein blocked the DNA binding and transcriptional activity of CCAAT/enhancer-binding protein β (C/EBP β) during differentiation by promoting the expression of C/EBP homologous protein, a dominant-negative member of the C/EBP family. Loss of C/EBP β activity was manifested as a loss of differentiation-induced C/EBP α and peroxisome-proliferator-activated receptor γ protein expression and a dramatic reduction

in lipid accumulation. Further, we documented for the first time that C/EBP β was tyrosine-phosphorylated *in vivo* during differentiation and *in vitro* by activated epidermal growth factor receptor. Genistein inhibited both of these events. Collectively, these results indicate that genistein blocks adipogenesis and C/EBP β activity by increasing the level of C/EBP homologous protein and possibly by inhibiting the tyrosine phosphorylation of C/EBP β .

Key words: adipocyte, daidzein, peroxisome-proliferator-activated receptor γ (PPAR γ), preadipocyte.

INTRODUCTION

In white adipose tissue, preadipocytes exist in close proximity to adipocytes and respond to positive energy balance by proliferating and then differentiating into adipocytes [1,2]. Mature adipocytes then accommodate excess energy through enhanced triacylglycerol storage. Many of the advances in our understanding of adipogenesis are based on studies in murine 3T3-L1 cells [3]. As preadipocytes, 3T3-L1 cells resemble fibroblasts. They replicate in culture until they form a confluent monolayer, after which cell–cell contact triggers G₀/G₁ growth arrest, an apparent prerequisite for subsequent differentiation into adipocytes [4]. Stimulation with 1-methyl-3-isobutylxanthine (MIX), dexamethasone and high doses of insulin in serum-containing medium (MDI protocol) for 2–3 days prompts these cells to undergo postconfluent mitotic clonal expansion, exit the cell cycle and commit to terminal differentiation (reviewed in [5]).

Adipogenesis involves co-operative interaction between members of the CCAAT/enhancer-binding protein (C/EBP) and peroxisome-proliferator-activated receptor (PPAR) families, the primary adipogenic transcription factors. C/EBP β , expressed immediately after the induction of differentiation, acquires DNA-binding capacity by 12–16 h [6] and dominates in inducing the initial expression of both PPAR γ and C/EBP α on days 2 and 3 of differentiation. PPAR γ and C/EBP α then induce the expression of each other and act synergistically to promote adipogenesis by transactivating the promoters of several adipocyte-specific genes [7,8].

Adipogenesis also requires the down-regulation of repressive factors, such as preadipocyte factor-1 [9], Wnts [10] and GATA factors [11], which maintain preadipocytes in the undifferentiated state. Several members of the C/EBP family also function as repressors of differentiation by forming inactive heterodimers with C/EBP β and C/EBP α . C/EBP homologous

protein [CHOP; also known as growth arrest and DNA damage-inducible gene 153 (GADD153)] functions as a dominant-negative inhibitor of the DNA-binding activity of C/EBP β and C/EBP α [12]. Similarly, heterodimers containing C/EBP β and either C/EBP γ or liver inhibitory protein, the truncated isoform of C/EBP β , attenuate the transcriptional activity of C/EBP β (reviewed in [13]). C/EBP α undifferentiated protein differs from the repressive C/EBPs in that it is expressed only by preadipocytes and it blocks transactivation of the C/EBP α gene promoter not by forming an inactive heterodimer but by occluding the binding site of a transcriptional activator [14].

Since the discovery in 1987 that genistein functions as a tyrosine kinase inhibitor [15], authors of more than 2500 published papers have used genistein to gauge tyrosine kinase activity in numerous cell lines. Wang and Malbon [16] have reported that genistein blocks 3T3-L1 cell differentiation in a dose-dependent manner. We [17] have found that genistein inhibits the proliferation of preconfluent preadipocytes, as well as the mitotic clonal expansion of postconfluent preadipocytes and their subsequent differentiation into adipocytes. However, when added on day 3 of differentiation, genistein has no inhibitory effect on adipogenesis. Given that the timing of genistein's anti-adipogenic effect coincides with the expression of C/EBP β , C/EBP α and PPAR γ , we hypothesized that genistein inhibits 3T3-L1 cell differentiation by preventing key tyrosine phosphorylation events involved in the expression or activation of these adipogenic transcription factors.

We report here that genistein increased CHOP expression, blocked the DNA binding and transcriptional activity of C/EBP β and prevented the subsequent expression of C/EBP α and PPAR γ . Because daidzein, which is structurally similar to genistein but inactive as a tyrosine kinase inhibitor, had no adverse effect on adipogenesis, we examined whether a tyrosine phosphorylation event regulates C/EBP β activity. We found, for

Abbreviations used: ALLN, *N*-acetyl-Leu-Leu-norleucinal; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; FBS, foetal bovine serum; GADD153, growth arrest and DNA damage-inducible gene 153; MIX, 1-methyl-3-isobutylxanthine; MDI, MIX, dexamethasone and insulin; NP40, Nonidet P40; PPAR, peroxisome-proliferator-activated receptor.

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the first time, that both MDI and activated epidermal growth factor (EGF) receptor induced the tyrosine phosphorylation of C/EBP β and that genistein inhibited these events.

MATERIALS AND METHODS

Materials

All tissue culture materials were from Gibco (Grand Island, NY, U.S.A.). Genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone) and activated EGF receptor tyrosine kinase were from Calbiochem (San Diego, CA, U.S.A.). Insulin, dexamethasone and MIX were from Sigma. Protein A/G-agarose, GADD153 fusion protein, anti-C/EBP α (14AA), anti-C/EBP β (H-7), anti-GADD153 (B-3) and anti-PPAR γ (E-8) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-phosphotyrosine (PY20) antibody was from PharMingen-Transduction Laboratories (Lexington, KY, U.S.A.). Anti-phosphotyrosine (4G10) antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The enhanced chemiluminescence (ECL) detection kit, horseradish peroxidase conjugated secondary antibodies and [γ - 32 P]ATP were from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Luciferase assay reagents were from BD Pharmingen (San Diego, CA, U.S.A.).

Cell culture

3T3-L1 preadipocytes (A.T.C.C., Manassas, VA, U.S.A.) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (10000 units/ml penicillin, 10000 μ g/ml streptomycin in 0.85% saline) and 1% (v/v) 100 μ M pyruvate at 37 °C in 95% air, 5% CO $_2$. To induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5 mM MIX, 0.5 μ M dexamethasone and 10 μ g/ml insulin (MDI) added to DMEM/10% FBS culture medium. On day 3, the MDI medium was replaced with DMEM/10% FBS, which was changed every 2 days thereafter until analysis. Isoflavones were reconstituted as 100 mM stock solutions in DMSO, filter-sterilized and stored at -20 °C. For each experiment, cells received isoflavones diluted in MDI differentiation medium.

Oil Red O staining

3T3-L1 adipocytes were washed with PBS (5% sucrose), fixed with 10% glutaraldehyde in PBS (pH 7.4), and stained with 0.5% Oil Red O.

Preparation of whole-cell lysates

3T3-L1 cells were washed twice in ice-cold PBS with 1 mM Na $_3$ VO $_4$, and then placed immediately in SDS-lysis buffer [1% Nonidet P40 (NP40), 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% NaN $_3$, 10 μ g/ml aprotinin, 1 μ M pepstatin, 16.4 μ g/ml leupeptin, 1 mM PMSF, 0.1 mM Na $_3$ VO $_4$, 2% SDS, 10% glycerol] without dithiothreitol (DTT) or tracking dye. Samples were heated for 10 min at 95 °C before protein concentrations were determined using the Bio-Rad DC protein determination kit.

Preparation of nuclear extracts

3T3-L1 cells were washed twice in PBS, once in hypotonic lysis buffer (20 mM Tris/HCl, 10 mM NaCl and 3 mM MgCl $_2$), and then scraped in hypotonic lysis buffer containing 1 mM DTT, 2 μ l/ml Pic I (1 mg/ml leupeptin, 1 mg/ml antipain

10 mg/ml benzamidine and 1 mg/ml aprotinin) and 2 μ l/ml Pic II (1 mg/ml chymostatin, 1 mg/ml pepstatin A in DMSO). A 1:100 volume of 10% NP40 was added, and cells were lysed by Dounce homogenization. After centrifugation for 5 min at 2200 rev./min (4 °C), the supernatant was discarded and the crude nuclear pellet was washed in hypotonic lysis buffer containing protease inhibitors, resuspended in nuclear storage buffer [40% glycerol, 50 mM Tris (pH 8.8), 3 mM MgCl $_2$, 1 mM DTT, 2 μ l/ml Pic I, 2 μ l/ml Pic II], and transferred to a microcentrifuge tube. Pelleted nuclei were then resuspended in 10 $\times$ volume of NUN buffer [1.1 M urea, 0.33 M NaCl, 17.5 mM Hepes (pH 7.6), 1.1 mM DTT, 2 μ l, ml Pic I, 2 μ l/ml Pic II], vortex-mixed for 5 s, incubated on ice for 30 min and centrifuged for 10 min at 14000 rev./min (4 °C) in a microcentrifuge. The supernatant was transferred to a fresh microcentrifuge tube and glycerol was added (10% final volume). Samples were diluted 10 times in water before protein concentrations were determined, using the Bio-Rad DC protein determination kit. Nuclear extracts were stored at -80 °C. Nuclear extracts obtained by this method contain no cytoplasmic proteins, as confirmed by the absence of heat-shock protein-90 [18] by immunoblot analysis using anti-heat-shock protein-90 (Transduction Laboratories) (results not shown).

Immunoblot analysis

Before adding 100 mM DTT and tracking dye, protein concentrations of whole-cell lysates or nuclear extracts were determined using the Bio-Rad DC protein determination kit. BSA was used as the standard. Samples were heated for 5 min at 95 °C, separated by SDS/PAGE (10% gel), and analysed by immunoblotting as described previously [19,20]. Immunoblots were developed with the ECL $^{\text{®}}$ kit.

Immunoprecipitation

Cells were placed in ice-cold gentle-lysis buffer [25 mM Tris/HCl (pH 7.5), 1% NP40, 10% glycerol, 50 mM NaF, 10 mM NaH $_2$ PO $_4$, 137 mM NaCl, 2 mM Na $_3$ VO $_4$, 1 mM PMSF, 10 μ g/ml aprotinin] and rocked on ice for 30 min. The lysed cells were harvested and centrifuged at 6000 g for 20 min at 4 °C. Supernatants were removed and protein concentrations were determined. For immunoprecipitations, PY20 antibody (10 μ g/ml) was added to lysates, which contained equal amounts of protein (700 μ g); incubation occurred overnight at 4 °C. Protein A/G-agarose beads were mixed with the immunoprecipitates on a rocker for 1 h at 4 °C. Immunoprecipitates were recovered by centrifugation at 2500 g and washed three times with ice-cold gentle-lysis buffer containing protease inhibitors. Immunoprecipitated proteins were dissolved in 2 $\times$ sample buffer and analysed by SDS/PAGE as described above.

Plasmids and transfection

Two-day postconfluent (d 0) 3T3-L1 preadipocytes were transiently co-transfected with calcium phosphate-precipitated DNA containing 5 μ g of a C/EBP α promoter-luciferase construct [21] and 5 μ g of a C/EBP β expression vector [22] provided by M. Daniel Lane (Johns Hopkins University School of Medicine). Four hours after transfection, cells were glycerol-shocked (12.5% glycerol in 1 $\times$ PBS, filter-sterilized) for 2 min and then placed in MDI differentiation medium plus 0.1% DMSO vehicle or 100 μ M genistein. After 48 h, cells were washed once with PBS, scraped in lysis buffer containing 100 mM KPO $_4$ (pH 7.8), 1% Triton X-100, 1 mM DTT and 2 mM EDTA, and stored at -20 °C until analysis for luciferase activity (Enhanced Luciferase

Assay Kit; PharMingen), which was normalized to that of undifferentiated day 0 cells (transfection but no MDI stimulation).

Electrophoretic mobility-shift assay

Nuclear extracts from 3T3-L1 cells induced to differentiate for 36 h in the presence of vehicle or 100 μ M genistein were prepared as outlined above and stored at -80°C . Protein concentrations were determined using the Bio-Rad DC protein determination kit. A [γ - ^{32}P]ATP-labelled (Amersham) double-stranded oligonucleotide probe (20 bp) corresponding to the C/EBP binding site in the C/EBP α promoter (GCG TTG CGC CAC GAT CTC TC, nt 191–172 [23]) was used for gel shift assays. The binding reaction mixture contained approx. 100000 c.p.m. of labelled DNA, 2 μ g of poly(dI/dC) and 10 μ g of cell extract protein in 30 μ l of buffer (10 mM Hepes/0.1 mM EDTA/5% glycerol/100 mM NaCl/0.3 M urea/0.3% NP40); this mixture was incubated on ice for 30 min. Where indicated, 0.4 μ g of anti-C/EBP β antibody (Santa Cruz) was added to the binding reaction. The reactions were analysed by 5% PAGE in 0.5 $\times$ Tris/borate/EDTA buffer, followed by autoradiography.

In vitro kinase assay

Protein lysates from 3T3-L1 cells differentiated for 0 or 24 h were subjected to immunoprecipitation as described above using 3 μ g of C/EBP β antibody/ml of lysate, containing 1 mg of protein. Immunoprecipitates were washed three times with NP40 lysis buffer [1% NP40/20 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 mM EDTA/0.1% Na N_3], then twice with KA buffer (50 mM Hepes (pH 7.6)/0.1 mM EDTA/0.015% Brij 35). Active EGF receptor kinase in KD buffer [50 mM Hepes (pH 7.6), 0.1 mM EDTA, 0.015% Brij 35, 0.1 mg/ml BSA, 0.2% 2-mercaptoethanol] was then added (approx. 1 unit/reaction), followed by approx. 5 μ Ci of [γ - ^{32}P]ATP in ATP buffer [50 mM Hepes (pH 7.6)/0.1 mM EDTA/0.015% Brij 35/30 mM MgCl $_2$ /0.15 mM ATP]. Samples were heated at 30°C for 30 min. After the addition of loading dye, samples were heated at 100°C for 2 min, separated by SDS/PAGE (10–15% gel), transferred to a nitrocellulose membrane, and analysed by autoradiography.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. The significance of differences between means, set at $P < 0.05$, was assessed by Student's t test (Microsoft Excel). Unless noted otherwise, reported results were reproduced in two additional independent experiments.

RESULTS

Genistein inhibits the expression of C/EBP α and PPAR γ , but has no effect on C/EBP β expression

Previous studies have demonstrated that genistein inhibits 3T3-L1 adipogenesis [16,17]. To determine whether genistein's anti-adipogenic effects result from inhibition of tyrosine kinase activity, we differentiated 3T3-L1 cells in the presence of genistein or daidzein. Although the structure of daidzein is nearly identical with that of genistein, with the exception of one critical hydroxyl group (Figure 1A), daidzein has no inhibitory effect on tyrosine kinase activity [15]. As shown in Figure 1(B), genistein dramatically inhibited lipid accumulation, compared with the vehicle control. Daidzein treatment, on the other hand, had little or no inhibitory effect. To determine the point in the differentiation

Figure 1 Effect of genistein versus daidzein on 3T3-L1 cell differentiation

(A) Structures of genistein and daidzein. (B) Two-day postconfluent 3T3-L1 cells (day 0) were induced to differentiate for 72 h in the presence of vehicle, 100 μ M genistein or 100 μ M daidzein. After 72 h, the differentiation medium was replaced with DMEM/10% FBS. On day 8 after induction of differentiation, cells were stained with Oil Red O.

programme where genistein exerts its anti-adipogenic effects, we examined the expression pattern of three transcription factors critical for adipogenesis, C/EBP β , C/EBP α and PPAR γ . Approximately 4 h after the induction of differentiation, 3T3-L1

Figure 2 Effect of genistein on the expression of adipogenic transcription factors

Two-day postconfluent 3T3-L1 cells induced to differentiate for 72 h in the presence of vehicle, 100 μ M genistein or 100 μ M daidzein were lysed on the indicated days for immunoblot analysis with antibodies against C/EBP β , C/EBP α and PPAR γ , all at a dilution of 1:200.

Figure 3 Effect of genistein on C/EBP β activity

Two-day postconfluent 3T3-L1 cells were induced to differentiate in the presence of vehicle (V) or 100 μ M genistein (G) and nuclear extracts were prepared, as outlined in the Materials and methods section. (A) Immunoblot analysis of nuclear extracts shows nuclear translocation of C/EBP β 36 h after MDI compared with 0 time (no MDI stimulation) or a control (CT) whole-cell lysate. (B) Electrophoretic mobility-shift assays were performed using a radiolabelled oligonucleotide probe corresponding to the C/EBP binding site in the C/EBP α promoter. Binding reactions were performed in the presence of anti-C/EBP β where indicated. The same results were obtained in another independent experiment. (C) To assess transcriptional activity of C/EBP β , a luciferase assay was performed, as outlined in the Materials and methods section. Relative luciferase activity indicates the fold increase over the average light intensity emitted by transfected but unstimulated control cells. The results reflect the means $\pm$ S.E.M. for three independent experiments; * P < 0.002, significantly different from vehicle. (D) Lysates obtained at the indicated times were immunoblotted with anti-C/EBP α antibody. The membrane was stripped and re-probed with anti-C/EBP β antibody to show equal protein loading.

cells express C/EBP β [6]. After a 24–48 h delay, C/EBP β triggers the expression of both PPAR γ and C/EBP α , which then promote the expression of each other and a number of adipocyte-specific genes (reviewed in [7]). As shown in Figure 2, genistein had no effect on MDI-induced C/EBP β expression, but dramatically inhibited the expression of both C/EBP α and PPAR γ . As expected, daidzein had no effect on the expression of C/EBP β , C/EBP α or PPAR γ .

Genistein inhibits C/EBP β activity

The results in Figure 2 indicate that genistein blocks adipogenesis downstream of C/EBP β expression but upstream of C/EBP α and PPAR γ expression. Because the initial expression of both C/EBP α and PPAR γ relies on C/EBP β activity, we determined whether C/EBP β is transcriptionally active in the presence of genistein. Although genistein had no effect on C/EBP β translocation to the nucleus (Figure 3A), its presence markedly impaired the ability of C/EBP β to bind an oligonucleotide corresponding to the C/EBP site in the C/EBP α promoter (Figure 3B). Because impaired DNA binding should also impair transcriptional activity, we examined whether genistein inhibits C/EBP β -induced transactivation of C/EBP α . We transiently co-transfected cells with a C/EBP α promoter-luciferase construct and a C/EBP β expression vector, induced differentiation for 48 h in the presence of vehicle or genistein, and analysed the cell lysates for luciferase activity. Figure 3(C) shows that genistein inhibited C/EBP β -induced transactivation of the C/EBP α promoter by approx. 60% compared with the vehicle control. Figure 3(D) shows a time course of MDI-induced C/EBP β and

Figure 4 Effect of genistein on CHOP expression

Two-day postconfluent 3T3-L1 cells were induced to differentiate in the presence of vehicle or 100 μ M genistein, and day 0–day 2 whole-cell lysates (A) or 36 h nuclear extracts from two independent experiments (B) were immunoblotted with an antibody against CHOP (GADD153) (1:100). This antibody also recognizes a 57 kDa tagged fusion protein corresponding to amino acids 1–168 of GADD153, which was included as a positive control (CT). The nuclear extract membrane was stripped and re-probed with an antibody against C/EBP β .

C/EBP α expression in the presence of vehicle or genistein. In both vehicle- and genistein-treated cells, C/EBP β remained at high levels throughout day 3 and declined by day 4. Although C/EBP α expression increased dramatically on day 3 in vehicle-treated cells, its expression remained low in genistein-treated cells despite high levels of C/EBP β .

Genistein increases CHOP expression

The basic DNA-binding region of CHOP contains proline and glycine substitutions for asparagine, aliphatic and basic residues that are conserved in other members of the C/EBP family. These substitutions prevent CHOP–C/EBP heterodimers from binding a number of C/EBP consensus sites, including adipose-specific sites [12]. By dimerizing avidly with C/EBP β and C/EBP α , CHOP functions as a transcriptional inhibitor of adipogenesis that blocks the differentiation of 3T3-L1 preadipocytes when ectopically expressed [24] or when up-regulated by the protease inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN) [23].

To determine whether inhibition of C/EBP β DNA binding by genistein results from increased expression of CHOP, we differentiated 3T3-L1 preadipocytes for 0–48 h in the presence of vehicle or genistein and analysed whole-cell lysates (Figure 4A) and nuclear extracts (Figure 4B) for CHOP expression. Treatment with genistein, but not vehicle, dramatically increased the level of CHOP.

Genistein inhibits the MDI-induced tyrosine phosphorylation of C/EBP β

Although many researchers have demonstrated the importance of serine and threonine phosphorylation in regulating C/EBP β

Figure 5 Effect of genistein on the tyrosine phosphorylation of C/EBP β

Day 0 cells were differentiated in the presence of vehicle or 100 μ M genistein and lysed at the indicated times for (A) immunoprecipitation (IP) with 10 μ g/ml anti-phosphotyrosine antibody (PY20), followed by immunoblotting (IB) with anti-C/EBP β antibody. The membrane was stripped and re-probed with anti-phosphotyrosine antibodies (PY20/4G10, 1:1000 each). The control lane (CT) contains a non-immunoprecipitated whole-cell lysate from cells differentiated for 24 h. For the *in vitro* kinase assay (B), protein lysates from day 0 cells differentiated for 24 h in the presence of vehicle or 100 μ M genistein were immunoprecipitated with 3 μ g of C/EBP β antibody/ml of lysate containing 1 mg of protein. Active EGF receptor kinase and [γ - 32 P]-labelled ATP were added. Samples were heated, separated by SDS/PAGE (10–15%), transferred to a nitrocellulose membrane and analysed by autoradiography. Membranes containing immunoprecipitated C/EBP β were stripped and re-probed with anti-C/EBP β antibody.

activity (reviewed in [25]), no published studies have reported that C/EBP β undergoes tyrosine phosphorylation in response to MDI stimulation. Because genistein is a tyrosine kinase inhibitor that blocks adipogenesis and C/EBP β activity, we examined whether C/EBP β undergoes tyrosine phosphorylation during adipogenesis and whether genistein inhibits this process. Immunoprecipitation of tyrosine-phosphorylated proteins from cells stimulated with MDI plus vehicle or genistein for 2 or 3 days, followed by immunoblotting with an antibody against C/EBP β , showed that MDI induced the tyrosine phosphorylation of C/EBP β (both the 34 and 38 kDa isoforms) on day 3 of differentiation and that genistein inhibited this event (Figure 5A). Stripping and re-probing the membrane with antibodies against tyrosine-phosphorylated proteins (PY20/4G10) confirmed that on day 3 of differentiation, vehicle-treated cells, but not genistein-treated cells, contained 34 and 38 kDa tyrosine-phosphorylated proteins.

Although serine and threonine kinases phosphorylate residues in specific consensus sites, tyrosine kinases are relatively promiscuous [26]. Not knowing the identity of the kinase that tyrosine phosphorylates C/EBP β , we speculated that the EGF receptor, which has intrinsic tyrosine kinase activity [27], would *in vitro* phosphorylate C/EBP β . Figure 5(B) shows that genistein inhibited the tyrosine phosphorylation of C/EBP β by the active EGF receptor kinase.

DISCUSSION

In the present study, we demonstrated that genistein disrupts the adipogenic programme by blocking C/EBP β activity, which in turn prevents the expression of both C/EBP α and PPAR γ , key adipogenic transcription factors. Several researchers have established that in preadipocyte cell lines, adipogenesis is driven by the following cascade of transcription factors: C/EBP β and C/EBP δ , expressed soon after the induction of differentiation, synergistically promote the expression of both C/EBP α and PPAR γ , the primary adipogenic transcription factors [28]. In 3T3-L1 cells, disruption of this cascade blocks the differentiation programme [29]. In addition, primary embryonic fibroblasts derived from C/EBP β / δ knockout mice neither differentiate nor express C/EBP α and PPAR γ in response to MDI stimulation [30]. These results indicate that C/EBP β and C/EBP δ function as the primary inducers of C/EBP α and PPAR γ in cell culture. We examined the effect of genistein on C/EBP β activity, not on C/EBP δ activity, because, of the two, C/EBP β functions as the dominant isoform [29]. We found that genistein had no inhibitory effect on C/EBP δ expression (results not shown). Additionally, Figure 3(B) shows that in the presence of genistein, no C/EBP isoform binds to the C/EBP site, suggesting that genistein inhibits C/EBP δ activity as well as C/EBP β activity. Thus genistein may function as the equivalent of a C/EBP β / δ knockout in terms of the activity of these transcription factors.

We found that genistein impairs the ability of C/EBP β to bind to DNA. Importantly, our results showed that genistein increases the expression of CHOP, a dominant-negative C/EBP isoform that readily forms heterodimers with C/EBP β and prevents C/EBP β from binding to DNA [12]. Our findings agree with those of Tang and Lane [23], who demonstrated that ALLN-induced up-regulation of CHOP during the first 48 h of MDI stimulation blocks C/EBP β DNA binding, C/EBP α expression and subsequent differentiation without affecting C/EBP β expression. Although Tang and Lane did not determine how ALLN induces CHOP expression (or prevents turnover of the CHOP message), changes in cell cycle proteins may be involved. For example, ALLN blocks mitotic clonal expansion and differentiation of 3T3-L1 cells by preventing the turnover of the cyclin-dependent kinase inhibitor p27 [31]. Genistein also inhibits mitotic clonal expansion [17] but did not inhibit the MDI-induced degradation of p27 (results not shown) that normally occurs on days 1 and 2 of differentiation [31,32]. Thus we speculate that the anti-adipogenic effects of genistein and ALLN result from different mechanisms. Further studies are needed to determine how genistein regulates CHOP expression.

The isoflavone daidzein resembles genistein, but possesses no activity as a tyrosine kinase inhibitor [15]. The finding that daidzein had minimal to no adverse effect on adipogenesis suggests that anti-adipogenic effects of genistein result from the inhibition of critical tyrosine phosphorylation events. Importantly, our reciprocal immunoprecipitation and *in vitro* kinase studies showed for the first time that C/EBP β becomes tyrosine-phosphorylated during differentiation and that genistein blocks this event. C/EBP β becomes tyrosine-phosphorylated on day 3 of differentiation, which correlates with the timing of C/EBP β -induced C/EBP α expression. Tyrosine phosphorylation, a well-known mechanism for protein activation [33], may therefore regulate the transcriptional activity of C/EBP β . Although our results showed that C/EBP β is tyrosine-phosphorylated during adipogenesis, we cannot rule out the possibility that MDI induces the tyrosine phosphorylation of other proteins that co-immunoprecipitate with C/EBP β . However, it is highly unlikely that the proteins would have exactly the same molecular masses as

the C/EBP β isoforms (34 and 38 kDa) and produce a similar expression pattern during adipogenesis.

We have not yet identified the tyrosine residues on C/EBP β that become phosphorylated during adipogenesis. C/EBP β has ten tyrosine residues, seven of which are located in the N-terminal transactivation domain. C/EBP β also has 20 serine residues located throughout the protein. In a previous study [25], several of these serines were identified as sites of regulation via phosphorylation, but no one has suggested a role for tyrosine phosphorylation in the regulation of C/EBP β activity. Extensive mutagenesis studies will be necessary to identify the regulatory tyrosines on C/EBP β and the specific combinations of phosphoserines and phosphotyrosines required for optimal activity of this transcription factor.

Genistein has proven to be a useful tool for advancing our understanding of cell signalling in the field of adipogenesis. By identifying CHOP and C/EBP β as targets of genistein, however, we have raised the possibility that pharmacological levels of genistein, currently under investigation as a treatment for breast and prostate cancer [34], may produce unwanted effects in several other tissues. For example, liver, lung and intestine, which also express high levels of C/EBP β , rely on this transcription factor for normal cell growth and differentiation [35]. Animal and human studies should therefore be conducted to determine the effects of genistein on liver, lung, intestine and adipose tissue.

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