Genistein Downregulates SREBP-1 Regulated Gene Expression by Inhibiting Site-1 Protease Expression in HepG2 Cells

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Abstract

Genistein is one of the most abundant isoflavones in soy. The effects of genistein on cholesterol synthesis and fatty acid oxidation have been well documented, but the effect of genistein on fatty acid synthesis remains unclear. Thus, we investigated the effect of genistein on fatty acid synthase (FAS) expressions in HepG2 cells. In HepG2 cells treated with 10 μmol/L genistein, mRNA and protein expressions of FAS, as well as FAS activity, were significantly decreased. The promoter region of FAS contains binding sites for the transcription factor called sterol regulated element binding protein 1 (SREBP-1); SREBP-1 must be processed by site-1 (S1P) and site-2 proteases to be activated. We also investigated the effects of genistein on S1P, SREBP-1 expression, and subsequent SREBP-1 processing by S1P in HepG2 cells. Genistein reduced the expression of S1P and the processing of SREBP-1 but did not change the expression of SREBP-1 mRNA.

Introduction

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is one of the most abundant isoflavones in soy. It is derived from the hydrolysis of the glycoside form in soy products and has cholesterol-lowering properties in both animals and humans (1,2). The relation between genistein and cholesterol metabolism has been studied at the cellular level: genistein inhibits cellular cholesterol synthesis and cholesterol esterification in HepG2 human hepatoma cells (3,4). Genistein also affects fatty acid oxidation. It exerts antidiabetic and hypolipidemic effects through the upregulation of the PPAR-regulated gene expressions (5). In a previous study, we found that genistein upregulates the expression and activity of CPT1A, a rate-limiting enzyme in the β-oxidation pathway, in HepG2 cells (6).

As described above, the effects of genistein on cholesterol synthesis and fatty acid oxidation are well known, but the effect of genistein on fatty acid synthesis has not yet been identified. To study the effects of genistein on fatty acid synthetic pathways, we investigated the effect of genistein on fatty acid synthase (FAS) expressions. FAS is the multifunctional protein that synthesizes the saturated fatty acid palmitate from acetyl CoA, malonyl CoA, and NADPH (7). FAS plays a central role in de novo fatty acid synthesis and in the long-term regulation of lipogenesis (8).

The promoter region of FAS contains a sterol regulatory element (SRE) (9). SRE is the target of 3 basic helix-loop-helix leucine zipper transcription factors called sterol regulatory element binding proteins (SREBP) (10). The SREBP belongs to the basic helix-loop-helix-leucine zipper family of transcription factors. They are synthesized as inactive precursors bound to the endoplasmic reticulum. To act as a transcription factor, the NH2 terminal domain of the SREBP must be cleaved in a 2-step proteolytic process by site-1 (S1P) and site-2 (S2P) proteases. The S1P cleaves the SREBP and initiates the translocation process to the nucleus of the active fragments of the SREBP. This proteolytic release of SREBP stimulates lipid synthesis in hepatocytes and other cells (10).

There are 3 forms of SREBP: SREBP-1a, SREBP-1c, and SREBP-2. Both SREBP-1a and SREBP-1c are encoded by a single gene using alternative transcription start sites that produce alternate forms of exon (10–12). In the liver, SREBP-1c and SREBP-2 are the predominant isoforms. SREBP-1c is responsible for the regulation of fatty acid biosynthetic genes and SREBP-1a is a potent activator of all SREBP-responsive genes (10).
SREBP-1c increases the expression of genes involved in the fatty acid synthetic pathway, such as FAS, stearoyl CoA desaturase-1 (SCD1), glycerol-3-phosphate acyltransferase (GPAT), and acetyl CoA carboxylases ([ACC] ACC1 and ACC2) (12). SREBP-2 is a relatively selective activator of cholesterol synthesis in liver and adipose tissue of mice (13). Therefore, the cleavage of SREBP-1 by the S1P is the first step in regulating the expression of the lipid synthetic gene; however, the effects of genistein on regulation of S1P and SREBP-1 processing are not yet known.

To further study the effects of genistein on the fatty acid synthetic pathways, we investigated the effects of genistein on the expression of S1P and SREBP-1 processing in HepG2 cells. We also examined the expressions of the SREBP-1 regulated genes, such as SCD1, GPAT, and ACC. 

Materials and Methods

Materials. Genistein, daidzein, malonyl-CoA, NADPH, cholesterol, 25-hydroxycholesterol, and SU5416 were purchased from Sigma Chemical. Fetal bovine serum was obtained from Hyclone. All other cell culture supplies, as well as Triazol, were purchased from Life Technologies.

Cell culture. The human hepatoma cell line HepG2 was used in all experiments. The HepG2 cells were grown in high glucose DMEM containing 10% fetal bovine serum, 100,000 U/L penicillin, and 100 mg/L streptomycin in 5% CO2 atmosphere at 37°C. Cells were seeded in 75 flasks at a density of 1.0 x 10⁶ cells per flask and grown for 24 h.

Real-time reverse transcriptase-PCR. The HepG2 cells were incubated for 24 h with serum-free DMEM and the media was replaced by serum-free DMEM containing 10 μmol/L genistein, 10 μmol/L daidzein, or 10 μmol/L SU5416. The cells were incubated for 24 h. The control cells were incubated in serum-free DMEM without genistein, daidzein, or SU5416 for 24 h. Daidzein is an isoflavone compound without a tyrosine kinase inhibitor activity. SU5416 is an indole compound that inhibits the tyrosine kinase activity (14). The chemically treated or untreated HepG2 cells were rinsed in PBS and harvested by scraping. Total RNA of the HepG2 cells was prepared with Triazol (Life Technologies). Complementary DNA was synthesized and amplified from −1 μg total RNA with a reverse transcription system (Promega). We preformed quantitative PCR with a Quantitect Probe PCR kit (QIAGEN). The primer and probe for FAS (assay ID: Hs00188912_m1), S1P (assay ID: Hs00188866_m1), SREBP-1 (assay ID: Hs00231674_m1), SCD1 (assay ID: Hs00748952_s1), GPAT (assay ID: Hs00612646_m1), ACC1 (assay ID: Hs00167385_m1), and ACC2 (assay ID: Hs00153715_m1) were obtained from an assay-on-demand system (Applied Biosystems). PCR was performed according to the method described by Mullen et al. (15). Anti-S1P, anti-SREBP-1, anti-SREBP-2, and anti-SCD1 were purchased from Santa Cruz Biotechnology, Lab Vision, BD Bioscience, and Alpha Diagnostic, respectively. We used anti-Hsp 70 (0.5 mg/L) to assess the loading of the protein. The ratio of mature form to immature form was determined by measuring the band intensity using ImageMaster 2D (Amersham Pharmacia Biotech).

FAS activity assay. We incubated HepG2 cells for 24 h with serum-free DMEM and replaced the media with serum-free DMEM containing 10 μmol/L genistein. The cells were then incubated for a further 24 h. The cells for the control group were incubated in serum-free DMEM for 24 h without genistein. The HepG2 cells were rinsed with PBS and homogenized by sonication in an extraction buffer: 0.1 mol/L potassium phosphate (pH 7.0), 0.1 mmol/L EDTA, and 1 mmol/L DTT. The supernatants were obtained by centrifugation at 1000 × g for 30 min were used as samples in the assays. The FAS activity was measured according to the method described by Uchiyama et al. (16). We measured the activity of FAS in HepG2 cells by following the decrease in absorbance at 340 nm, resulting from the oxidation of NADPH.

Statistical analysis. All measurements were performed independently in at least triplicate. Results are expressed as means ± SD. Statistical analyses were performed using Student’s t-test or 1-way ANOVA with post hoc Dunnett’s tests for comparisons between 2 groups or multiple groups, respectively. Differences were considered significant at P < 0.05.

Results

FAS expression and activity. Genistein (10 μmol/L, 24 h) significantly decreased the FAS mRNA level and FAS enzyme activity (Table 1), as well as FAS protein expression (Fig. 1), compared with untreated HepG2 cells.

S1P expression. In cells treated for 24 h with 10 μmol/L genistein, mRNA expression of S1P was lower than in untreated cells (P < 0.01) (Fig. 2). In the 10 μmol/L daidzein treated cells, the mRNA level of S1P did not differ from that of the control cells. In cells treated with 10 μmol/L SU5416, S1P mRNA expression was greater than in untreated cells (P < 0.01) (Fig. 2). The expressions of the S1P protein were also significantly lower when the HepG2 cells were treated with 10 μmol/L and 50 μmol/L genistein than in untreated HepG2 cells (Fig. 3).

Effect of genistein on SREBP-1 and SREBP-2 mRNA and proteins. We investigated the effects of genistein on SREBP-1 at the mRNA and protein levels. In genistein treated cells, the

<table>
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<tr>
<th>Genistin</th>
<th>mRNA</th>
<th>FAS</th>
<th>SCD1</th>
<th>ACC1</th>
<th>ACC2</th>
<th>GPAT</th>
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<td>μmol/L</td>
<td>fold of control</td>
<td>nmol NADPH/min⁻¹</td>
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<td>0.58 ± 0.11</td>
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<tr>
<td>10</td>
<td>0.30 ± 0.06²</td>
<td>0.51 ± 0.09²</td>
<td>0.21 ± 0.04²</td>
<td>0.32 ± 0.15²</td>
<td>0.25 ± 0.02²</td>
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Table 1: Effect of genistein on mRNA level of SREBP-1 regulated genes and activity of FAS in HepG2 cells incubated with 10 μmol/L genistein for 24 h

¹ Values are means ± SD, n = 3. Asterisks indicate different from control: *P < 0.01, **P < 0.05.
mRNA level of SREBP-1 was similar to that of the control cells (data not shown). To study the effect of genistein on SREBP-1 and SREBP-2 processing, we used an immunoblot assay to determine the contents of mature and immature SREBP-1 and SREBP-2 proteins in the HepG2 cell nucleus. Amounts of 10 μmol/L and 50 μmol/L genistein decreased the ratio of mature to immature SREBP-1, compared with that of the control. On the other hand, 50 μmol/L genistein increased the ratio of mature to immature SREBP-2, and cholesterol and 25-hydroxycholesterol treatments decreased the ratio of mature to immature SREBP-1, compared with the control groups (Fig. 4).

We found that genistein affects the proteolytic cleavage of SREBP-1 rather than the SREBP-1 mRNA expression in HepG2 cells.

**Effect of genistein on the expression of SCD1.** To examine the effect of genistein on SCD1 mRNA and expression in HepG2 cells, we measured the mRNA level of SCD1 using real time PCR. After the genistein treatment, the mRNA level of SCD1 in HepG2 cells was significantly lower than that in the untreated control (Table 1). To investigate the effect of genistein on SCD1 protein expression, we performed an immunoblot analysis with the HepG2 cells after the genistein treatment. Genistein decreased SCD1 protein expression significantly, compared with untreated HepG2 cells (Fig. 5).

**Effect of genistein on the mRNA expression of ACC1, ACC2, and GPAT.** To determine whether genistein inhibits the mRNA expression of ACC1, ACC2, and GPAT, we measured their mRNA levels using real time PCR. Genistein inhibited the mRNA expression of ACC1, ACC2, and GPAT significantly, compared with the untreated control (Table 1).

**Discussion**

Genistein shows a hypolipidemic action by suppressing the hepatic lipid synthesis in glomerulonephritic rats (17). Genistein also suppresses fatty acid synthesis in isolated rat adipocytes (18). In the fatty acid synthetic pathway, FAS is the rate-limiting enzyme (19). In this study, we investigated the effects of genistein on the regulation of FAS in HepG2 cells. When HepG2 cells were cultured in the presence of genistein, FAS expression and enzyme activity were significantly lower than those of the control cells.

The transcription of the FAS gene is regulated by SREBP-1, and SREBP-1 should be cleaved by S1P and S2P to bind the SREBP-1 regulated gene promoter. Thus, we examined the effect of genistein on the expression of S1P expression in HepG2 cells.
With genistein, the mRNA and protein levels of S1P were reduced in HepG2 cells. To determine whether the inhibition effect of genistein resulted from the estrogenic activity or the tyrosine kinase activity, we observed mRNA levels of S1P in daidzein-treated cells and SU5416-treated cells. Daidzein is an isoflavone compound without a tyrosine kinase inhibitor activity and SU5416 is a general inhibitor of receptor tyrosine kinases. Daidzein did not change the mRNA level of S1P, but SU5416 increased the mRNA level of S1P. These results suggest that the inhibition of S1P mRNA expression by genistein resulted from neither the estrogenic effect nor the tyrosine kinase inhibitor activity. It appears to result from some other activity of genistein.

SU5416, a receptor tyrosine kinase inhibitor, increased the expression of the S1P mRNA. The promoter region of S1P gene contains binding motifs for Sp1 (20). The activation of Sp1 transcriptional activity results from the increase in protein phosphatase 2A (PP2A) activity. The catalytic subunit of PP2A is inactivated by phosphorylation of the Tyr307 residue by the receptor tyrosine kinase (21). We suggest that SU5416 activates PP2A by inhibiting the phosphorylation of Tyr307 residue. The PP2A activated by SU5416 may enhance the transcriptional activity of Sp1 and result in the increase of S1P mRNA expression.

To further study how genistein inhibits S1P expression, we investigated the promoter sequence of S1P using the TFSEARCH program (22). The promoter sequence of S1P was reported by Nakajima et al. (20). A computer analysis using the TFSEARCH program revealed that the promoter sequence of S1P contains several binding motifs for AML-1a. AML-1a is also found in the promoter region of the osteopontin gene (23). mRNA expression of osteopontin is decreased by genistein, but not by herbimycin A, the tyrosine kinase inhibitor in UMR106–06 osteoblast-like cells (24). Therefore, this genistein effect on the expression of S1P may result from the AML-1a binding motif in the S1P gene promoter. However, the molecular mechanism underlying the inhibition of S1P expression by genistein remains to be clarified. The effect of genistein on the promoter of the S1P gene and upstream signals should be investigated.

We also investigated the effect of genistein on SREBP-1 expression and activation processing. To act as a transcription factor, the amino terminal of SREBP-1 must be released from the membrane by S1P and S2P (10). We found that inhibiting S1P expression with genistein resulted in the inhibition of the proteolytic activation of SREBP-1, rather than the inhibition of SREBP-1 mRNA expression. S1P and S2P are involved in the processing of another protein, ATF6. ATF6 is a membrane-bound transcription factor, similar to SREBP-1 (25). ATF6 is cleaved to act as a transcription factor for the expression of genes, such as GRP78, GRP94, and calnexin, which encode chaperone molecules that restore the folding of proteins in the endoplasmic reticulum (26). Genistein has been found to inhibit the expression of GRP78 by azetidin (27). One possible mechanism of GRP78 inhibition by genistein could be that genistein inhibits the ATF6 processing, which is required for the transcription of GRP78, by suppressing the expression of S1P. The effect of genistein on the processing of ATF6 should also be examined.

The regulation of the SREBP-1 pathway by genistein can differ from that of the SREBP-2 pathway. We suggest that, in the SREBP-2 pathway, genistein can be an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase or other enzymes in the cholesterol biosynthetic pathway; furthermore, inhibition of enzymes in the cholesterol synthetic pathway effects an increase of the mature SREBP-2 protein (15). In this study, we also found that the mature form of SREBP-2 was increased by the genistein treatment. In the SREBP-2 pathway, the cholesterol level is a key factor in regulating SREBP-2 cleavage (28). The cleavage of SREBP-2 by genistein is primarily affected, not by the regulation of S1P expression, but by the downregulation of the cholesterol level by inhibiting the cholesterol synthetic enzymes.

Transcription of genes for fatty acid synthesis is regulated by SREBP-1 (10). SCD1 is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids. The monounsaturated fatty acids synthesized by SCD1 are the major substrates for the synthesis of various lipids, such as phospholipids, triglycerides, and cholesterol esters (29). The transcription of SCD1 is inhibited by polyunsaturated fatty acids and cholesterol. The promoter region of SCD1 contains a SRE and the transcription of SCD1 is regulated by SREBP-1 (30). In a cDNA microarray analysis, the expression of SCD1 was downregulated by genistein in MCF-7 cells (31). GPAT catalyzes the triacylglycerol synthesis by the acylation of the sn-1 hydroxyl of glycerol-3-phosphate. The expression of GPAT is regulated by SREBP-1 (32). Acetyl-CoA carboxylases (ACC1 and ACC2) catalyze the carboxylation of acetyl-CoA to malonyl-CoA. Malonyl CoA is the substrate for fatty acid biosynthesis, as well as the suppressor of fatty acid β-oxidation. The regulations of ACC are mediated by SREBP-1 (33). The expression levels of SCD1, GPAT, and ACC were significantly reduced by the genistein treatment. Genistein inhibited the expressions of SCD1, GPAT, and ACC by inhibiting the S1P expression followed by the inhibition of SREBP-1 maturation.

In conclusion, this study revealed that genistein inhibited S1P expression, which resulted in an inhibition of the SREBP-1 activation process and consequent downregulation of SREBP-1 regulated genes, such as FAS, SCD1, ACC, and GPAT in HepG2 cells.

### Literature Cited


