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JNK-Mediated SREBP-2 Processing by Genistein up-regulates LDLR Expression in HepG2 Cells

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Abstract

Genistein has been implicated for its anti-atherogenic effects. We investigated the molecular mechanisms behind the impact of genistein on expression of LDLR, the receptor for LDL-cholesterol, and related signaling pathways in HepG2 cells. Genistein increased mRNA and protein levels of LDLR in a time-dependent manner. In order to find out the effects of genistein on the transcriptional levels, a luciferase reporter construct containing LDLR promoter (pLDLR-luc) was constructed and examined for its response to genistein. Genistein increased the reporter activity but failed to increase transcriptional activity when sterol-regulatory element (SRE) in the LDLR promoter was deleted. Genistein increased nuclear translocation of SREBP-2 and DNA binding activity of SREBP-2 to LDLR promoter by chromatin immunoprecipitation assay (CHIP). Pre-treatment of 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), serine protease inhibitor, prevented the effects of genistein while brefeldin A causing the fusion of the endoplasmic reticulum (ER) and the Golgi apparatus did not, suggesting that genistein may have an effect on SREBP-2 trafficking from the ER to the Golgi apparatus. Insig-1 protein levels were not changed by genistein. Among mitogen-activated protein kinases (MAPK), genistein phosphorylated JNK but not p38 and ERK signals. JNK inhibitor (SP600126) abolished genistein-stimulated levels of LDLR and nuclear SREBP-2. To minimize the effects of c-Jun, a transcription factor activated by JNK signals, a truncated LDLR luciferase construct that contained SRE but lacked the c-jun putative binding site was constructed. Genistein still was able to boost the transcriptional activity of the truncated LDLR construct. All the genistein effects were abolished by the addition of cholesterol. In conclusion, genistein has the anti-atherogenic effects by activating JNK signals and SREBP-2 processing, which is followed by up-regulation of LDLR. However, the beneficial effects of genistein could be affected by the amount of cellular cholesterols.

Keywords: Genistein; JNK; LDL receptor; Cholesterol; SREBP-2

Abbreviations: LDLR: Low Density Lipoprotein Receptor; SREBP-2: Sterol Regulatory Element Binding Protein-2; JNK: c-Jun N-Terminal Kinase

Introduction

Hypercholesterolemia is a strong risk factor for the development of atherosclerosis, a hallmark of cardiovascular disorders [1]. Familial hypercholesterolemia suggests that hepatic expression of LDLR, a receptor for LDL-cholesterol, is crucial for the regulation of blood cholesterol levels [2,3] . Extensive studies indicate that statins improve lipid profiles by inhibiting the activity of HMGCR, a rate-limiting enzyme of cholesterol synthesis, thereby increasing expression of LDLR [4]. Even so, statins only reduce cardiovascular risk by about 20 to 40% [5]. Diet alone successfully lowers blood LDL-cholesterol to a degree comparable to statin treatment [6]. Combination therapy using statins and a dietary regime may further reduce blood cholesterol levels [5]. Moreover, the neutral antagonist treatment of type 1 cannabinoid receptor may improve blood lipid profile [7,8].

Sterol regulatory element-binding protein 2 (SREBP-2) is a key transcription factor in cholesterol metabolism. SREBP-2 regulates the transcription of LDLR for cellular uptake of LDL cholesterol. At a high concentration of sterol, precursor SREBPs (pre-SREBPs) complex with SCAP and Insig proteins and are retained in the endoplasmic reticulum (ER) [9,10]. At a low concentration of sterol, Insig proteins are no longer bound to SCAP and the pre-SREBP complex, causing them to translocate to the Golgi apparatus. There, pre-SREBPs undergo proteolytic cleavage by serine proteases, resulting in liberation of the N-terminal region, which is a nuclear SREBP. Nuclear SREBP enters the nucleus and binds a sterol-regulatory element (SRE) in the promoter region of target genes such as LDLR [11-14]. SREBP-regulated gene expression has its own negative intrinsic regulatory mechanism.

Dietary soy isoflavones, including genistein, have received great attention as anti-atherogenic foods due to their lipid-improving effects [15-19], but *in vivo* and *in vitro* findings regarding their total and/or LDL cholesterol-lowering effects have been inconsistent [20-25]. Some studies report that dietary supplementation with genistein, a strong bioactive soy isoflavone, lowers total and LDL-cholesterol levels [23,24], while others describe no effect of soy isoflavones on the concentration of LDL-cholesterol even when vascular function is improved [25].

In the current study, we sought to understand the molecular actions of genistein on the expression of LDLR in hepatocytes and implications for observed hypocholesterolemic or anti-atherogenic effects. Although LDLR is expressed in nearly all tissues, liver LDLR plays a pivotal role in the clearance of LDL-cholesterol [26].

Materials and Methods

Cell culture

Human hepatoblastoma (HepG2) cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and grown in 5% CO_2 at 37°C in high glucose DMEM medium (Welgene, Daegu, Korea) containing

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10% (v/v) FBS (Cellgro, Manassas, VA, USA) and 5% AA (Invitrogen, Carlsbad, CA, USA). For experiments, cells were plated at a density of 1.6×10^6 cells per 60mm dish. The next day, the medium was changed to a serum-starvation medium containing 0.5% (v/v) FBS. After overnight incubation, the cells were treated with genistein at a final concentration of 10 μM or 20 μM for the indicated periods of time. For JNK inhibition experiments, cells were pre-treated with the JNK inhibitor SP600126 (Calbiochem, Darmstadt, Germany) at a concentration of 10 μM for 30 min prior to the addition of genistein. The inhibitors 0.3 μM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) (Sigma, St. Louis, MO, USA), 1 μg/ml brefeldin A (Sigma, St. Louis, MO, USA), and 10 μM MG132 (Calbiochem,Darmstadt, Germany) were used in our study.

Plasmids and cloning

A previously described reporter plasmid pSRE-luc was a gift from Dr. Shimano [27]. A luciferase reporter gene plasmid containing the LDLR promoter region was constructed. A promoter fragment of the human LDLR gene from -805 to +50 relative to the transcription start site was amplified from genomic DNA extracted from HepG2 cells by PCR using the following primers: forward 5'- AACTCGAGTTGGTC-TCCACCAGCTCTCT-3' and reverse 5'- TGAAGCTTTCACGAC-CTGCTGTGTCCTA -3'. The PCR product was subcloned into the XhoI and HindIII sites of the pGL3basic luciferase vector (Promega, Madison, WI, USA) to generate pLDLR-luc (-805 to +50). Using the parental clone (pLDLR-luc (-805 to +50)), a 5' deletion construct containing the region from -171 to +50 (pLDLR (-171 to +50)) of the human LDLR gene was generated by PCR using the following primers: forward 5' – AACTCGAGGGACTGGAGTGGGAATCAGA- 3' and reverse 5'- TGAAGCTTTCACGACCTGCTGTGTCCTA -3'. SRE deletion constructs of pLDLRdelSRE-luc (-805 to +50) and pLDLRdelSRE (-171 to +50) were generated using the following primers: forward 5'-TGAAGACATTTGAAATGCAAACTCCTCCCCCTGCT-3' and reverse 5'- GGGGAGGAGTTTGCATTTCAAATGTCTTCACCTCAC-TGC-3'.

Transient transfection and luciferase assay

HepG2 cells were seeded in 6-well plates (Corning Costar Corp., Tewksbury, MA, USA) at 1.6×10^5/ well 24 h prior to transfection. Transfections were performed with 0.5 μg of each DNA construct and pRLSV40 (Promega, Madison, WI, USA) using Lipofectamin2000 (Life Technologies, Inc., Grand Island, CA, USA) according to the manufacturer's instructions. Twenty-four hours later, genistein was added to the medium. The next day, the cells were washed 3 times in PBS and lysed with 100 μl of passive lysis buffer. The clear cell lysate was used for the measurement of luciferase activity using the Dual-luciferase assay system (Promega, Madison, WI, USA) and a Promega Glomax luminometer (Promega BioSystems Sunnyvale Inc., Sunnyvale, CA, USA).

RNA extraction and quantitative RT-PCR

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from samples according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA after being primed by random hexamers through reverse-transcription by ImprompII reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's directions. Quantitative PCR was performed on a CFX96 sequence detection system (Biorad, Hercules, CA, USA) using EvaGreen qPCR mix plus (Solis BioDyne, Estonia). Levels of expression were normalized relative to the amount of 18S rRNA. Relative mRNA levels were calculated by differences in $\mathbf{C}_t$ values and are expressed as the fold change.

Western blot analysis

HepG2 cells were harvested and lysed in RIPA buffer containing leupeptin, 1mM PMSF, Na_3VO_4, and 0.1% protease inhibitor cocktails (Sigma, St. Louis, MO, USA). After 30 min incubation on ice, the cells were centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was mixed with 5X sample buffer and then boiled for 10 min. Equal amounts of protein cell lysates were loaded onto an SDS-polyacrylamide gel for electrophoresis. PVDF membrane (Millipore, Billerica, MA, USA) was used to transfer proteins from the gel. Mouse anti-SREBP-2 (Santa Cruz Biotech., Santa Cruz, CA, USA), rabbit anti-LDLR (BioVision, Milpitas, CA, USA), rabbit anti-p-JNK (Merck Millipore, Billerica, MA, USA), rabbit anti-p-p38 (Merck Millipore, Billerica, MA, USA), mouse anti-p-ERK (Santa Cruz Biotech., Santa Cruz, CA, USA), and rabbit anti-GAPDH (Signalway antibody, Pearland, TX, USA) antibodies were used to detect the proteins.

Chromatin immunoprecipitation assay (CHIP)

Cells were crosslinked with 0.4% ice cold formaldehyde (Sigma-Aldrich, MO, USA) for 15 min at room temperature. The reaction was stopped by adding 0.2M of glycine and incubating for 5 min at room temperature. After being rinsed with cold PBS twice, the cells were lysed with 250 μl RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), 5 mM EDTA). The lysates were sonicated 4 times, 30s each time, with a sonicator (Sonifier 250, Branson, CT, USA). After centrifugation at 13,000 rpm for 15 min at 4°C, the clear supernatant was used for immunoprecipitation. The pre-cleared protein lysates were gently rotated with anti-SREBP-2 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) overnight at 4°C and then rotated with a 50% slurry of blocked protein A/G sepharose beads (Santa Cruz, CA, USA) for 2h at 4°C. Samples were washed with RIPA buffer 2 times, IP buffer (100 mM Tris (pH 8), 500mM LiCl, 1% NP-40, 1% sodium deoxycholate) 4 times, RIPA buffer 2 times and TE buffer 2 times. Every wash process was carried out for 5 min at 4°C. IP material was eluted by adding 200 μl of elution buffer (70 mM Tris (pH 8), 1 mM EDTA, 1.5% SDS) in the remaining 100 μl of TE buffer (10 mM Tris, 1mM EDTA, pH 7.5) from the last wash step and incubating in a 65°C water bath for 10 min. Chromatin was reverse-crosslinked by adding 13 μl of 4M NaCl to 237 μl of the sample and incubating in a 65°C water bath for 5 h. DNA fragments were purified using a DNA extraction kit (iNtRON Biotechnology, Korea) and PCR was performed with the following primers: LDLR-SRE forward, 5'- TCCTCTTGCAGTGAGGTGAA-3', reverse, 5'- TTTCTAGCAGGGGGAGGAGT-3'. The PCR generated a 66-bp fragment containing a sterol regulatory element (SRE) of the human LDLR promoter.

Statistical Analyses

SPSS was used to perform statistical analyses (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test was used to determine statistically significant differences among the experimental groups. P values < 0.05 were considered statistically significant.

Results

Genistein increases LDLR expression

LDLR protein expression was examined in HepG2 cells after

incubation with varying concentrations of genistein. As the concentration increased from 1 μM to 20 μM, the protein levels of LDLR increased 1.8 fold to 2.9 fold (p<0.05) (Figure 1A). In time course experiments, a significant increase in LDLR mRNA levels was detected approximately 4 h after the addition of 10 μM genistein (Figure 1B). Significant elevation of LDLR mRNA levels was detected around 6 h of treatment with genistein (Figure 1C). Furthermore, when LDLR promoter activity was investigated using a luciferase construct containing the proximal region of the LDLR gene, genistein increased transcriptional activity of the construct (Figure 2A). These results suggest that genistein increases the expression of LDLR at the transcriptional level.

Genistein-induced expression of LDLR requires SREBP-2 transcriptional activity

We investigated whether SREBP-2 is involved in the genistein-mediated up-regulation of LDLR, based on the fact that SREBP-2 is a crucial transcription factor for the LDLR gene. In order to examine whether SREBP-2 transcriptional activity is required for genistein-mediated up-regulation of the LDLR gene, luciferase reporter constructs were generated. Genistein significantly increased luciferase activity, and this effect was counteracted by an SRE deletion construct (Figure 2A). The luciferase construct driven by SRE alone, which originated from the human SREBP-2 promoter region, responded to genistein by increasing luciferase activity (Figure 2B). In addition, the DNA binding activity of SREBP-2 upon genistein treatment was higher, as tested by CHIP (Figure 2C). These results clearly suggest that SREBP-2 transcriptional activity is required for the genistein-driven up-regulation of the LDLR gene.

Genistein promotes SREBP-2 processing

Protein levels of the mature and nuclear forms of SREBP-2 significantly increased within 6 h of genistein treatment (Figure 3A). Regulatory steps affected by genistein in the SREBP-2 maturation process were examined by the use of chemical inhibitors AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) and brefeldin A. AEBSF significantly lowered the nuclear form of SREBP-2 in both the absence and presence of genistein (Figure 3B), suggesting that the step regulated by genistein occurs prior to proteolytic cleavage in the Golgi apparatus. Brefeldin A increased nuclear SREBP-2 levels (Figure 3C). Genistein did not further elevate nuclear SREBP-2 levels beyond the increase caused by brefeldin A (Figure 3C). In addition, Insig-1 protein levels were not down-regulated within 12 h after genistein treatment, which presumably triggered SREBP-2 processing (Figure 3D). These data suggest that genistein may promote the movement of pre-SREBP-2 from the ER to the Golgi apparatus without significantly affecting Insig-1 degradation.

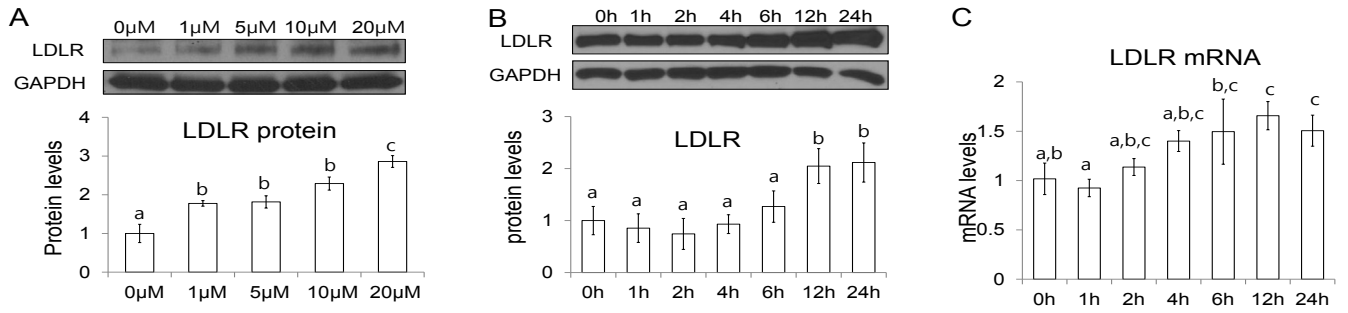

Figure 1: Genistein stimulation of LDLR expression at the transcriptional level. HepG2 cells were serum-starved and then stimulated with genistein. Protein levels of LDLR in response to 1, 5, 10 and 20 μM of genistein for 24 h (A), changes in protein (B) and mRNA levels (C) of LDLR after incubation with 10 μM of genistein within 24 h. Results are expressed as mean ± SD of at least three independent experiments. Different letters (a-c) represent significant differences (p<0.05) between all groups by ANOVA.

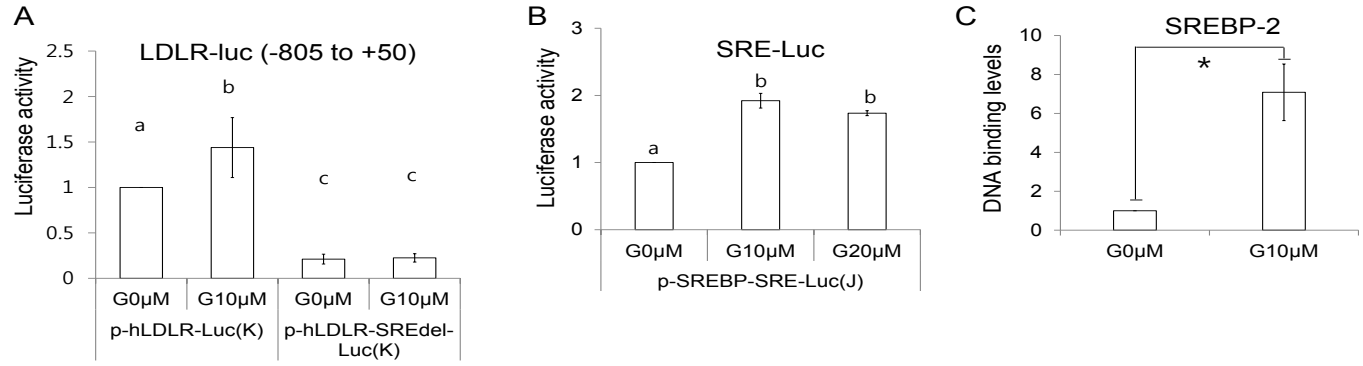

Figure 2: SRE-dependent effects of genistein on LDLR promoter activity.

Luciferase activities of phLDLR-luc(-805+50) and its corresponding SRE deletion construct (A) and pSRE-luc construct (B) upon the treatment of 10 or 20 μM genistein (G10 μM or G20 μM) and DMSO (G0 μM) for 24 h. CHIP assay for binding of SREBP-2 to SRE regions of the human LDLR promoter region (C). The results are expressed as mean ± SD of at least three independent experiments. Different letters (a-c) represent significant differences (p<0.05) for the comparison of all groups by ANOVA (A and B) or two groups by student's t-test (C).

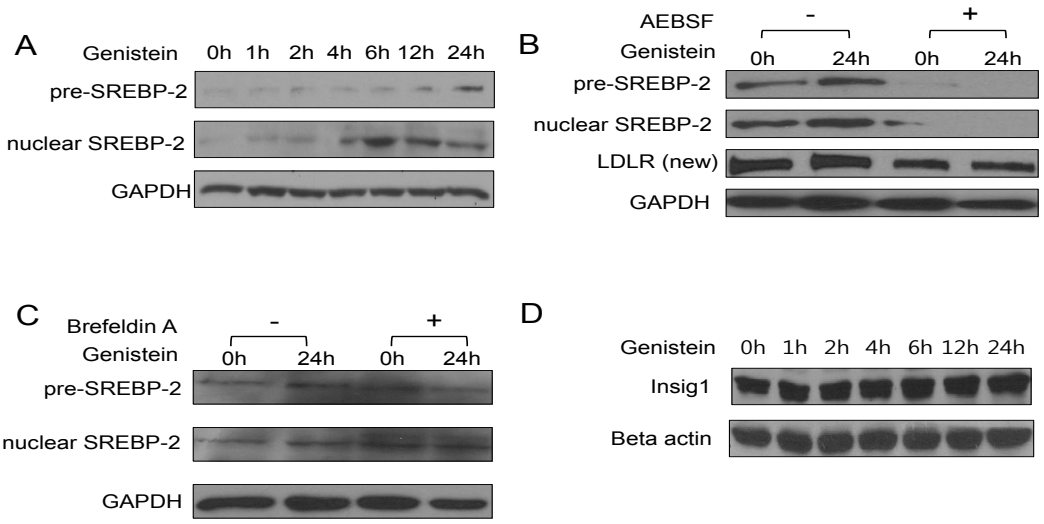

Figure 3: Genistein-stimulated SREBP-2 processing.

HepG2 cells were stimulated with genistein for the indicated period of times and analyzed by Western blot. Formation of nuclear SREBP-2 over time after genistein treatment (A), the effects of AEBSF (B) or brefeldin A (C) on the formation of nuclear SREBP-2 by genistein. Protein levels of Insig-1 over the course of genistein treatment for 24 h (D).

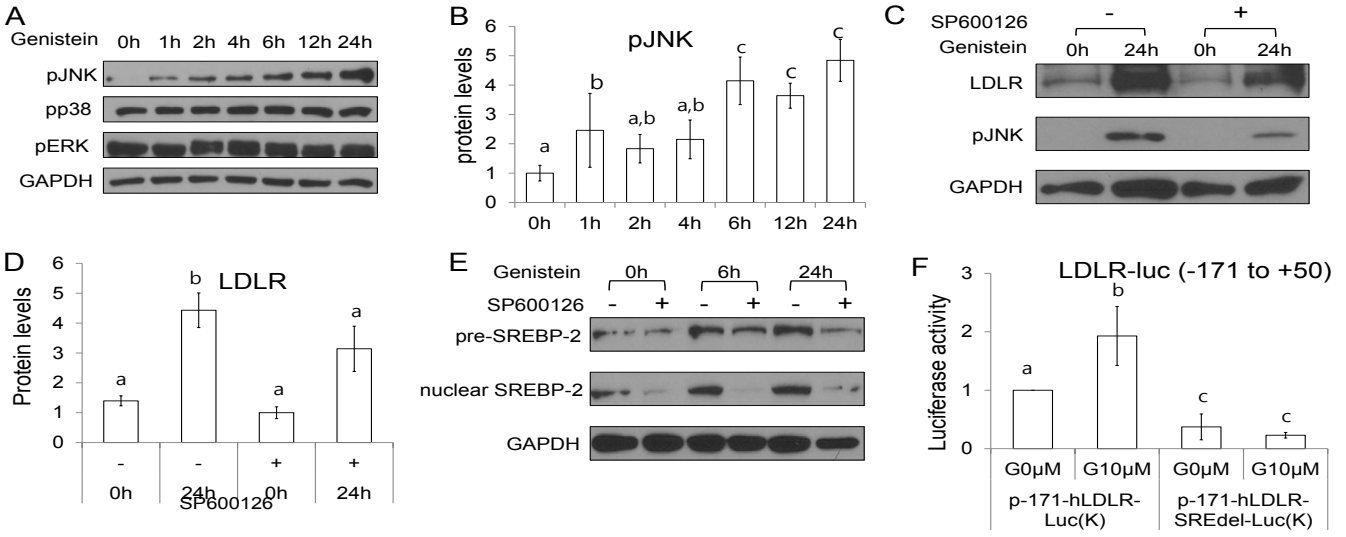

Figure 4: JNK activation by genistein involves SREBP-2 processing and LDLR expression.

Phosphorylated levels of JNK, p38 and ERK upon 10 μM genistein treatment for the indicated period of times (A) and quantification of p-JNK levels (B). LDLR and p-JNK protein levels with pre-treatment of SP600126 or vehicle DMSO (C) and quantification of LDLR protein levels (D). Protein levels of pre-SREBP-2 and nuclear SREBP-2 in cells treated with genistein for 6 or 24 h after 30 min incubation with SP600126 or DMSO (E). Luciferase activities of pLDLR-luc (-171 to +50) and pLDLRdelSRE-luc (-171 to +50) upon 24 h incubation of 10μM genistein (G10μM) or DMSO (G0μM). The results are expressed as mean ± SE of at least three independent experiments. Different letters (a-c) represent significant differences between all groups ($p<0.05$) by ANOVA.

Genistein activates the JNK signaling pathway for SREBP-2 processing

Next, we asked whether mitogen-activated protein kinase (MAPK) signaling pathways are involved in genistein-mediated LDLR expression. Genistein didn't significantly alter levels of the phosphorylated form of p38 and pERK proteins (Figure 4A), but phosphorylation of JNK protein increased gradually over the course of the 24 h treatment (Figure 4A and B). To assess if JNK is involved in the regulation of the LDLR gene by genistein, we treated cells with a JNK specific inhibitor, SP600126. SP600126 greatly reduced the phosphorylation of JNK and LDLR protein levels that had been increased by genistein (Figure 4C and D). In addition, pretreatment with SP600126 greatly lowered the levels of nuclear SREBP-2 (Figure 4E). In order to minimize the effects of c-jun, a transcription factor responding to JNK activation, we generated a construct harboring 171 base pairs of the upstream region of the human LDLR gene that did not contain the putative c-jun binding site but did have an SRE (pLDLR-luc (-171 to +50)). We also generated an SRE deletion construct. Genistein increased luciferase activity with the construct (pLDLR-delSRE-luc (-171 to +50)), and the SRE deletion

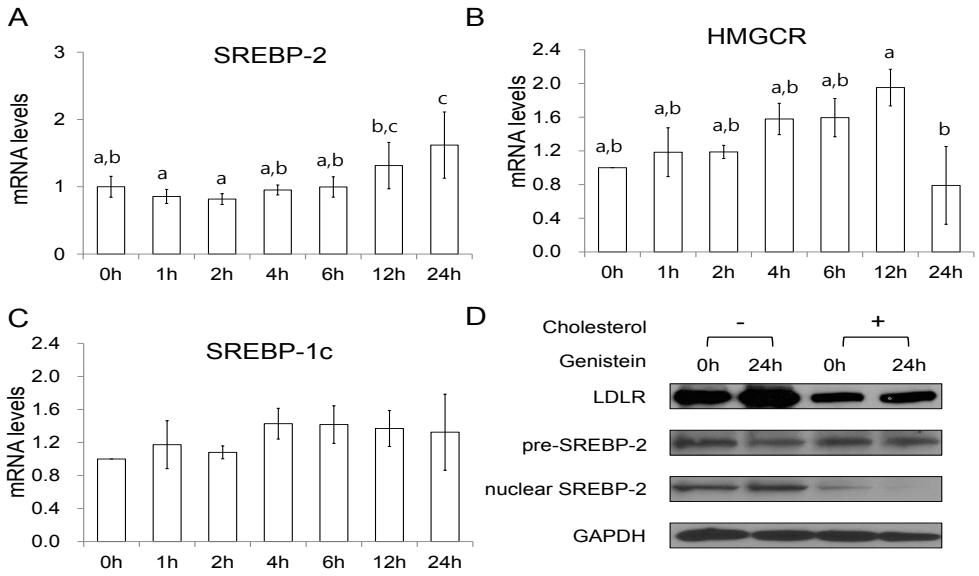

Figure 5: Transcript levels of SREBPs and HMGCR and cholesterol effects on nuclear SREBP-2 levels in genistein-treated cells.

mRNA levels of SREBP-2 (A), HMGCR (B) and SREBP-1c (C) in cells treated with 10 μM genistein for 24 h. Protein levels of LDLR, pre-SREBP-2, nuclear SREBP-2 and GAPDH in cells incubated with 10 μM of genistein in the presence of either cholesterol (5 μg/ml) or solvent alone (0.1% ethanol) for 24 h (D).

construct counteracted the effects of the genistein (Figure 4F). These results indicate that the activation of JNK signals is necessary for genistein-induced SREBP-2 maturation and, thereby, LDLR expression.

Genistein didn't up-regulate SREBP-1c mRNAs and cholesterol prevented genistein effects

Genistein didn't significantly up-regulate SREBP-2 mRNAs within 12 hr after the addition but 24 hr treatment of genistein appear to up-regulate SREBP-2 mRNA levels (Figure 5A). Although genistein markedly increased LDLR mRNA levels, 24 h incubation with geneistein didn't significantly up-regulate HMGCR mRNA levels (Figure 5B). SREBP-1c didn't change its expression levels in response to genistein (Figure 5C). Together, it is likely that there are other regulatory factors affecting the expression levels of HMGCR and SREBP-1c. Then we asked if cholesterol affects the regulation of genistein on SREBP-2 processing and LDLR expression. Cholesterol lowered nuclear levels of SREBP-2 and subsequently LDLR protein levels (Figure 5D). Even when the concentration of genistein was increased to 20 μM cholesterol abolished the genistein effect on SREBP-2 processing (data not shown). These data suggest that elevation in intracellular cholesterol concentration prevent the ability of genistein to promote SREBP-2 processing.

Discussion

In a previous animal study, the anti-atherogenic effects of genistein appeared to mediate LDLR based on a lack of the effects in LDLR knock-out mice [19], but the exact molecular mechanisms behind the actions of genistein were not investigated. Here, we demonstrate that genistein activates the JNK signaling pathway and increases SREBP-2 processing for SRE-dependent transcription of the LDLR gene in human hepatocytes. Because hepatic LDLR plays a crucial role in the regulation of blood LDL cholesterol, up-regulation of LDLR in hepatocytes by genistein is likely to contribute to its anti-atherogenic effects.

LDLR is regulated by various cellular stimuli such as growth factors, insulin [28] and estradiol [28,29]. SRE is required for insulin or estradiol- induced expression of the LDLR gene [28]. Maturation of SREBP by proteolytic cleavage has been implicated in regulation by growth factors such as platelet-derived growth factor [30]. Our data showed that genistein also up-regulates LDLR expression at transcriptional levels, which requires SRE-mediated transcriptional activity of SREBP-2.

The genistein-induced transcriptional activity of SREBP-2 appears to be due to an increase in the amount of nuclear SREBP-2. Nuclear localization of SREBP-2 can be regulated in at least two places: trafficking between the ER and Golgi apparatus and the proteolytic release of the nuclear form of SREBP-2 from the precursor form in the Golgi apparatus. We utilized AEBSF and brefeldin A to isolate the location of genistein-regulated SREBP-2 processing. AEBSF is a serine protease inhibitor that directly blocks the activity of Site-1 protease (S1P), a processing enzyme present in the Golgi apparatus [31]. Brefeldin A inhibits protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus, resulting in the retention of Golgi proteins, including the processing enzymes, in the ER [32,33]. Co-treatment with genistein and AEBSF yielded a lack of nuclear SREBP-2, while the addition of Brefeldin A did not alter the effects of genistein, suggesting that genistein promotes pre-SREBP-2 trafficking from the ER to the Golgi apparatus. Even so, we cannot completely exclude the possibility of genistein increasing the proteolytic cleavage of pre-SREBP-2 in the Golgi apparatus. Genistein-induced SREBP-2 processing is not likely to be mediated by Insig-1 degradation, because no significant changes in Insig-1 protein levels occurred upon genistein treatment. Genistein could have induced a structural change in the SREBP-SCAP complex that weakened interactive forces with Insig proteins to initiate movement to the Golgi.

Intracellular signaling mechanisms involved in soy isoflavone-mediated up-regulation of the LDLR gene have not been extensively elucidated. MAP kinase-related signals has been implicated in the transcriptional activation of SREBPs [34]. Here we provide evidence of the role of JNK activation on SREBP-2 processing and LDLR expression.

JNK inhibition greatly reduced genistein-increased nuclear SREBP-2 as well as its target gene expression of LDLR, suggesting that the JNK pathway links the effects of genistein and the activation of SREBP-2 for LDLR expression. Involvement of JNK activation in LDLR expression has been previously reported in berberine-treated cells [35]. However the effect of berberine on JNK activation and LDLR expression is SRE-independent and mediated by the transcriptional activity of c-jun, which is phosphorylated upon JNK activation [35]. Thus, our findings regarding JNK-dependent SREBP-2 activation of LDLR expression by genistein are novel. JNK activation is better understood in the regulation of SREBP-1c-mediated gene expression. JNK2 increases insulin-mediated activation of SREBP-1c for de novo fatty acid synthesis [34]. Another MAPK, ERK, mediates the signals initiated by insulin and platelet-derived growth factor [36]. ERKs directly phosphorylate nuclear SREBPs and lower the recruitment of SUMO-mediated HDAC, resulting in the enhancement of SREBP transcriptional activity [37].

SREBP-2 activation by genistein could result in expression of other target genes such as HMGCR and SREBP-2 itself. We observed that HMGCR mRNA levels were not significantly up-regulated by genistein, which may indicate that factors other than SREBP-2 as affected by genistein influence regulation of HMGCR and LDLR expression. SREBP-2 mRNA levels were elevated only at the time point of 24 h after genistein treatment, suggesting that a positive feedback loop of SREBP-2 enhanced SREBP-2 mRNA production. On the other hand SREBP-1c, which is under the control of sterol-regulated processing similar to that of SREBP-2, was not significantly up-regulated by genistein.

LDLR expression is controlled in a tight negative feedback mechanism by intracellular sterol levels [26]. The LDLR-lowering effects of genistein were sensitive to the presence of cholesterol. Cholesterol abolished genistein-induced SREBP-2 processing and LDLR expression. This outcome suggests that elevation of intracellular cholesterol concentration by genistein via LDLR could initiate a negative feedback mechanism to blunt the effects of the genistein. In addition, the sensitivity of genistein to the presence of cholesterol may indicate that genistein acts at the step at which sterol exerts its effect during SREBP-2 processing. Sterol prevents pre-SREBP-2 from trafficking from the ER to the Golgi apparatus by being associated with Insig-1 and SCAP proteins [38]. Because of plausible negative feedback mechanisms, the cholesterol-lowering effects of genistein could diminish over time, further limiting uptake of LDL-cholesterol elicited by genistein. This loop may explain the inconsistent ability of genistein to lower cholesterol.

In conclusion, our results demonstrate that genistein, a soy isoflavone, could act as an LDL-cholesterol-lowering agent via JNK activation and SREBP-2 processing in hepatocytes. The long-term hypocholesterolemic effects of genistein may require additional intervention to restrict the negative feedback mechanisms initiated by elevation of cholesterol levels.

Acknowledgments

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References

1. Grundy SM (1998) Hypertriglyceridemia, atherogenic dyslipidemia, and the metabolic syndrome. Am J Cardiol 81: 18B-25B.
2. Bilheimer DW, Goldstein JL, Grundy SM, Starzl TE, Brown MS (1984) Liver transplantation to provide low-density-lipoprotein receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. N Eng J Med 311:1658-1664.
3. Goldstein JL, Brown MS (1987) Regulation of low-density lipoprotein receptors: implications for pathogenesis and therapy of hypercholesterolemia and atherosclerosis. Circulation 76: 504-507.
4. Pedersen TR, Tobert JA (2004) Simvastatin: a review. Expert Opin Pharmacother 5: 2583-2596.
5. Thongtang N, Lin J, Schaefer EJ, Lowe RS, Tomassini JE, et al. (2012) Effects of ezetimibe added to statin therapy on markers of cholesterol absorption and synthesis and LDL-C lowering in hyperlipidemic patients. Atherosclerosis 225: 388-396.
6. Jenkins DJ, Kendall CW, Marchie A, Faulkner DA, Wong JM, et al. (2005) Direct comparison of a dietary portfolio of cholesterol-lowering foods with a statin in hypercholesterolemic participants. Am J Clin Nutr 81: 380-387.
7. Mastinu A, Pira M, Pinna GA, Pisu C, Casu MA, et al. (2013) NESS06SM reduces body weight with an improved profile relative to SR141716A. Pharmacol Res 74: 94-108.
8. Mastinu A, Pira M, Pani L, Pinna GA, Lazzari P (2012) NESS038C6, a novel selective CB1 antagonist agent with anti-obesity activity and improved molecular profile. Behav Brain Res 234: 192-204.
9. Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, et al. (2002) Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 110: 489-500.
10. Radhakrishnan A, Sun LP, Kwon HJ, Brown MS, Goldstein JL (2004) Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain. Mol Cell 15: 259-268.
11. Goldstein JL, DeBose-Boyd RA, Brown MS (2006) Protein sensors for membrane sterols. Cell 124: 35-46.
12. Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125-1131.
13. Jeong HJ, Lee HS, Kim KS, Kim YK, Yoon D, et al. (2008) Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. J Lipid Res 49: 399-409.
14. Dong B, Wu M, Li H, Kraemer FB, Adeli K, et al. (2010) Strong induction of PCSK9 gene expression through HNF1alpha and SREBP2: mechanism for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters. J Lipid Res 51: 1486-1495.
15. Lin Y, Meijer GW, Vermeer MA, Trautwein EA (2004) Soy protein enhances the cholesterol-lowering effect of plant sterol esters in cholesterol-fed hamsters. J Nutr 134: 143-148.
16. Taku K, Umegaki K, Sato Y, Taki Y, Endoh K, et al. (2007) Soy isoflavones lower serum total and LDL cholesterol in humans: a meta-analysis of 11 randomized controlled trials. Am J Clin Nutr 85: 1148-1156.
17. Hoie LH, Morgenstern EC, Gruenwald J, Graubaum HJ, Busch R, et al. (2005) A double-blind placebo-controlled clinical trial compares the cholesterol-lowering effects of two different soy protein preparations in hypercholesterolemic subjects. Eur J Nutr 44: 65-71.
18. Vitolins MZ, Anthony M, Burke GL (2001) Soy protein isoflavones, lipids and arterial disease. Curr Opin Lipidol 12: 433-437.
19. Kirk EA, Sutherland P, Wang SA, Chait A, LeBoeuf RC (1998) Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. J Nutr 128: 954-959.
20. Fukui K, Tachibana N, Wanezaki S, Tsuzaki S, Takamatsu K, et al. (2002) Isoflavone-free soy protein prepared by column chromatography reduces plasma cholesterol in rats. J Agric Food Chem 50: 5717-5721.
21. Demonty I, Lamarche B, Jones PJ (2003) Role of isoflavones in the hypocholesterolemic effect of soy. Nutr Rev 61: 189-203.
22. Weggemans RM, Trautwein EA (2003) Relation between soy-associated isoflavones and LDL and HDL cholesterol concentrations in humans: a meta-analysis. Eur J Clin Nutr 57: 940-946.
23. Squadrito F, Marini H, Bitto A, Altavilla D, Polito F, et al. (2013) Genistein in the metabolic syndrome: results of a randomized clinical trial. J Clin Endocrinol Metab 98: 3366-3374.
24. Irace C, Marini H, Bitto A, Altavilla D, Polito F, et al. (2013) Genistein and endothelial function in postmenopausal women with metabolic syndrome. Eur J Clin Invest 43: 1025-1031.

25. Nestel PJ, Yamashita T, Sasahara T, Pomeroy S, Dart A, et al. (1997) Soy isoflavones improve systemic arterial compliance but not plasma lipids in menopausal and perimenopausal women. Arterioscler Thromb Vasc Biol 17: 3392-3398.

26. Brown MS, Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. Science 232: 34-47.

27. Amemiya-Kudo M, Shimano H, Hasty AH, Yahagi N, Yoshikawa T, et al. (2002) Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. J Lipid Res 43: 1220-1235.

28. Streicher R, Kotzka J, Müller-Wieland D, Siemeister G, Munck M, et al. (1996) SREBP-1 mediates activation of the low density lipoprotein receptor promoter by insulin and insulin-like growth factor-I. J Biol Chem 271: 7128-7133.

29. Nanjee MN, Koritnik DR, Thomas J, Miller NE (1990) Hormonal determinants of apolipoprotein B,E receptor expression in human liver. Positive association of receptor expression with plasma estrone concentration in middle-aged/elderly women. Biochim Biophys Acta 1046: 151-158.

30. Demoulin JB, Ericsson J, Kallin A, Rorsman C, Ronnstrand L, et al. (2004) Platelet-derived growth factor stimulates membrane lipid synthesis through activation of phosphatidylinositol 3-kinase and sterol regulatory element-binding proteins. J Biol Chem 279: 35392-35402.

31. Okada T, Haze K, Nadanaka S, Yoshida H, Seidah NG, et al. (2003) A serine protease inhibitor prevents endoplasmic reticulum stress-induced cleavage but not transport of the membrane-bound transcription factor ATF6. J Biol Chem 278: 31024-31032.

32. Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 56: 801-813.

33. DeBose-Boyd RA, Brown MS, Li WP, Nohturfft A, Goldstein JL, et al. (1999) Transport-dependent proteolysis of SREBP: relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi. Cell 99: 703-712.

34. Ito M, Nagasawa M, Omae N, Tsunoda M, Ishiyama J, et al. (2013) A novel JNK2/SREBP-1c pathway involved in insulin-induced fatty acid synthesis in human adipocytes. J Lipid Res 54: 1531-1540.

35. Lee S, Lim HJ, Park JH, Lee KS, Jang Y, et al. (2007) Berberine-induced LDLR up-regulation involves JNK pathway. Biochem Biophys Res Commun 362: 853-857.

36. Kotzka J, Müller-Wieland D, Roth G, Kremer L, Munck M, et al. (2000) Sterol regulatory element binding proteins (SREBP)-1a and SREBP-2 are linked to the MAP-kinase cascade. J Lipid Res 41: 99-108.

37. Arito M, Horiba T, Hachimura S, Inoue J, Sato R (2008) Growth factor-induced phosphorylation of sterol regulatory element-binding proteins inhibits sumoylation, thereby stimulating the expression of their target genes, low density lipoprotein uptake, and lipid synthesis. J Biol Chem 283:15224-15231.

38. Sakai J, Nohturfft A, Goldstein JL, Brown MS (1998) Cleavage of sterol regulatory element-binding proteins (SREBPs) at site-1 requires interaction with SREBP cleavage-activating protein. Evidence from in vivo competition studies. J Biol Chem 273: 5785-5793.