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ORIGINAL CONTRIBUTION

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Positive regulation of hepatic carnitine palmitoyl transferase 1A (CPT1A) activities by soy isoflavones and L-carnitine

Summary *Background* Genistein increases CPT1A, a rate-limiting enzyme in the β-oxidation pathway, enzyme activity by increasing CPT1A transcription in HepG2 cells and, consequently, suppresses high fat induced obesity in C57BL/6J mice. Genistein and daidzein are the most abundant isoflavones in soy. *Aim of study* To investigate the effect of co-treatment of genistein and L-carnitine on CPT1A enzyme activity and to determine whether daidzein also increases CPT1A activity and to establish a cell line that can be used to screen chemicals to regulate CPT1A transcription. *Methods* The enzyme activities of CPT1A were determined after HepG2 cells were incubated with 10 μM genistein or 10 μM daidzein or 1 mM L-carnitine or in combination with 10 μM genistein and 1 mM L-carnitine or in combination with 10 μM daidzein and 1 mM L-carnitine. The mRNA expression levels of CPT1A were determined by real time PCR method after HepG2 cells were incubated with 10 μM genistein or 10 μM daidzein. A suggested CPT1A promoter region was cloned from human genomic DNA and the CPT1A promoter-luciferase reporter gene construct was made, and the promoter-reporter gene construct was transfected into human hepatoma cell line Huh7. *Results* The enzyme activity of CPT1A was at least 2.3-fold higher in L-carnitine and genistein co-treated HepG2 cells than either single-agent treated cells. Daidzein also significantly increased the mRNA expression of CPT1A as well as the enzyme activity of CPT1A. A stable Huh7 cell line, which was selected after Huh7 cells were transfected with CPT1A promoter luciferase reporter gene construct, was characterized by confirming that luciferase activity of the cell line can be regulated by genistein and daidzein as well as clofibrate, a well-known CPT1A mRNA up-regulating drug. *Conclusions* Genistein and daidzein can up-regulate CPT1A enzyme activity through up-regulation of CPT1A transcription. Co-treatment of L-carnitine and genistein additively increases CPT1A enzyme activity in HepG2 cells. A stable Huh7 cell line transfected with the CPT1A promoter luciferase reporter gene was established and characterized.

Key words carnitine palmitoyltransferase 1A – genistein – daidzein – L-carnitine – β-oxidation

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Introduction

Genistein and daidzein are the most abundant isoflavones in soy and derived from the hydrolysis of their glycoside forms present in soy products. They regulate the mRNA level of genes involved in cholesterol metabolism, such as hydroxymethylglutaryl-CoA (HMG-CoA) reductase, low-density lipoprotein (LDL) receptor in HepG2 cell [1]. However, the effects of isoflavones on β-oxidation have not been known although the β-oxidation is an important pathway for fatty acid metabolism.

Carnitine palmitoyltransferase 1 (CPT1) is at the outer mitochondrial membrane and catalyzes the long-chain fatty acid translocation into the mitochondrial

matrix [2]. It has been known that CPT1 plays a pivotal role in controlling long-chain fatty acid oxidation in mitochondria and acts as a rate-limiting enzyme [3]. It is also known that an increase in CPT1 activity results in an increase in fatty acid oxidation and energy expenditure at the cellular level and in the animal model [4, 5]. Therefore, it is widely accepted that the oxidation of long-chain fatty acids is regulated at the level of CPT1 through changes in CPT1 activity [2]. CPT1 has at least two isoforms; liver type CPT1 (CPT1A) and muscle type CPT1 (CPT1B) [6].

Changes in CPT1A activity occur in response to alterations in the nutritional and hormonal status of animal or cell. Both the mRNA level and activity of CPT1A are regulated by a high-carbohydrate diet in rat [6]. Fatty acids induced the mRNA expression of CPT1A in the pancreatic β-cell line INS-1. The gene expression of CPT1A was also regulated by glucagon and c-AMP [2]. The increase in CPT1A mRNA was followed by an increase in CPT1A enzyme activity [5]. If CPT1A expression and enzyme activity can be regulated by nutrients, the oxidation of long-chain fatty acids can be regulated by nutrients. We found that genistein increases CPT1A enzyme activity by increasing CPT1A transcription in HepG2 cells and, consequently, suppresses high fat induced obesity in C57BL/6J mice [7].

In this study, we investigated the effect of co-treatment of genistein and L-carnitine on CPT1A activity. The enzyme activity of CPT1A was at least 2.3-fold higher in L-carnitine and genistein co-treated cells than either of the single-agent treated cells. We have shown that daidzein also increases CPT1A enzyme activity by increasing CPT1A transcription. The organization of the human CPT1A gene was determined and promoter region was suggested by Gobin et al. [8]. In order to make a luciferase reporter gene assay system for human CPT1A promoter activity, the suggested CPT1A promoter region was cloned from human genomic DNA and CPT1A promoter-luciferase reporter gene construct was made, and a stable cell line was selected after the promoter-reporter gene construct was transfected into human hepatoma cell line Huh7.

Materials and methods

Materials

Clofibrate, daidzein, genistein, palmitoyl-coenzyme A (palmitoyl-CoA), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and L-carnitine were obtained from Sigma Chemical. Triton X-100 was obtained from Pharmacia Biotech. Fetal bovine serum (FBS) was obtained from Hyclone. All other cell culture supplies and Triazol were purchased from Life Technologies.

HepG2 Cell culture

HepG2 cells were grown in high glucose Dulbecco's Modified Eagle medium (DMEM) containing 10 % FBS, 100,000 U/L penicillin and 100 mg/L streptomycin in 5 % CO_2 atmosphere at 37 °C. For experiments, cells were seeded in T75 flasks at a density of 1.0×10^6 cells per flask and grown for 24 h. Then medium was replaced by serum-free DMEM. After incubation for another 24 h, medium was replaced by serum-free DMEM containing 10 μM genistein or 10 μM daidzein or 1 mM L-carnitine. Cells were incubated for designated periods and cells for the control group were incubated for the same periods in serum-free DMEM.

Determination of CPT1A activity

To determine the activity of CPT1A, HepG2 cells were harvested after 48-hour treatment with 10 μM genistein or 10 μM daidzein or 1 mM L-carnitine or in combination with 10 μM genistein and 1 mM L-carnitine or in combination with 10 μM daidzein and 1 mM L-carnitine. For activity assay, cell homogenates were prepared as described in the literature [9] and protease inhibitors (Protease inhibitor cocktail, Sigma) were added to cell homogenates. CPT1A activity in cell homogenates was assayed spectrophotometrically by following the release of CoA-SH from palmitoyl-CoA using the general thiol reagent DTNB; method for activity measurement was modified from method described by Karlic et al. [10]. Reaction mixtures containing DTNB buffer and cell homogenate were incubated at room temperature for 20 minutes to eliminate all the reactive thiol groups. After incubation, the absorbance was measured at 412 nm. To start reaction, palmitoyl-CoA (100 μM, final concentration) in double distilled water and L-carnitine solution (1 mM, final concentration in 1M Tris, pH 8.0) were added to reaction mixtures. Reaction mixtures were incubated for 10 min at 37 °C. After incubation, the absorbance was measured at 412 nm. The difference between absorbance with and without substrates measures the release of CoA-SH. Activity was defined as nmole CoA-SH released/min/mg protein. The protein content of the cell homogenates was determined according to the method of Bradford.

Real-time reverse transcriptase-PCR (RT-PCR)

HepG2 cells were incubated for 24 hours with 10 μM isoflavones, daidzein or genistein. Isoflavone-treated or -untreated HepG2 cells were rinsed in phosphate-buffered saline (PBS) and harvested by scraping. Total RNA of HepG2 cell was prepared with Triazol (Life Technologies). Complementary DNA was synthesized and

amplified from about 1 µg total RNA with TaqMan One Step PCR Master Mix Reagents Kit (Applied Biosystems). Primer and probe for CPT1A were obtained from Assay-on-Demand Gene Expression system (Assay ID: Hs00157079_m1, Applied Biosystems). Reaction and analysis were performed using Rotor-Gene 3000 system (Corbett Research). The relative amounts of all mRNAs were calculated by using the comparative C_T method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the invariant control.

CPT1A promoter luciferase reporter gene construct

A promoter fragment of the CPT1A 5′-untranslated region from –562 to +1890 relative to the transcription start site was amplified from human genomic DNA by PCR using oligonucleotides forward primer (gggggtaccccgaatgaatgaatgaacgaatg) and reverse primer (cggatcccgcgtgcgcataaaatcgc) [8]. The forward primer contained a *Kpn*I restriction site and the reverse primer contained a *BamH*I restriction site. The PCR product was subcloned into pCR-Blunt II-TOPO (Invitrogen, CA). pCR-Blunt II-TOPO-CPT1A promoter construct was digested by *Kpn*I and *BamH*I. The CPT1 promoter fragments were removed from pCR-Blunt II-TOPO-CPT1A promoter construct by digestion with *Kpn*I and *BamH*I and ligated into *Kpn*I and *Bgl*II sites of pGL3-Enhancer (Promega, WI) vector in front of luciferase reporter gene to generate pGL3-CPT1A.

Transfection of Huh7 cells

Huh7 cells were grown in DMEM medium supplemented with 10 % fetal bovine serum. For transfection of pGL3-CPT1A, cells were seeded in 6-well plate and cultured until 80 % confluence. Co-transfection was performed with 1 µg of construct DNA, 0.1 µg pcDNA 3.1 vector (Invitrogen). Huh7 cells were transfected using a standard LipofectAMINE method (Life Technologies, Inc.). Stably transfected cell lines were selected in the presence of 500 µg/ml G418 (active Geneticin antibiotic in distilled water, GIBCO) for 3 weeks with the culture medium changed three times a week. Individual G418-resistant colonies were clone-purified followed by measuring luciferase activity. Empty vector (pGL3-Enhancer) transfected cells were used as a negative control.

Luciferase assay

Cells for Luciferase assay were cultured in 96 well (2.0×10^5 cells/well). Luciferase activity was measured by the luciferase assay system with reporter lysis buffer (Promega) following the manufacturer's instruction. Cells were treated with lysis buffer for 15 min. Luciferase activity was measured using 1420 Victor multi label counter (Perkin Elmer). Measurement was performed in triplicate independently. Luciferase assays (relative light units, RLU) were normalized using the purchased recombinant luciferase (Promega) as the standard. Vector (pGL3-Enhancer)-transfected Huh7 cells were used as negative controls.

Statistical analysis

All measurements are performed at least in triplicate independently. Results are expressed as means ± standard deviation. Statistical significance was calculated with Student's t test.

Results

The effect of genistein, daidzein and L-carnitine on CPT1A activity

To investigate the effect of daidzein, genistein and L-carnitine treatment on CPT1A enzyme activity, the enzyme activity of CPT1A in HepG2 cell was measured using Ellman's reagent [11]. HepG2 cells were treated with 1 mM carnitine or 10 µM daidzein or 10 µM genistein for 48 h or in combination with 10 µM genistein and 1 mM L-carnitine or in combination with 10 µM daidzein and 1 mM L-carnitine for 48h and the enzyme activity of CPT1A was measured by following the release of CoA-SH from palmitoyl-CoA. As shown in Fig. 1, the enzyme activity of CPT1A in L-carnitine and genistein co-treated HepG2 cells was 6.3-fold higher than that of control cells and at least 2.3-fold higher than those of either single-agent treated cells [nmole CoA-SH released/min/mg protein]: 0.93 ± 0.25 (control) versus 2.56 ± 0.83 (genistein) versus 2.51 ± 0.51 (L-carnitine) versus 5.90 ± 0.73 (genistein/L-carnitine). Daidzein also increased the enzyme activity of CPT1A by 5.5-fold (Fig. 1). The enzyme activity of CPT1A in L-carnitine and daidzein co-treated HepG2 cells was 5.5-fold higher than that of control cells (Fig. 1).

The effect of genistein and daidzein on CPT1A mRNA transcription

The effect of daidzein and genistein on CPT1A mRNA expression in HepG2 cells was determined by RT-PCR method. When cells were cultured in medium containing 10 µM daidzein or 10 µM genistein for 24 h, the mRNA expression of CPT1A was significantly increased by both daidzein and genistein (Fig. 2).

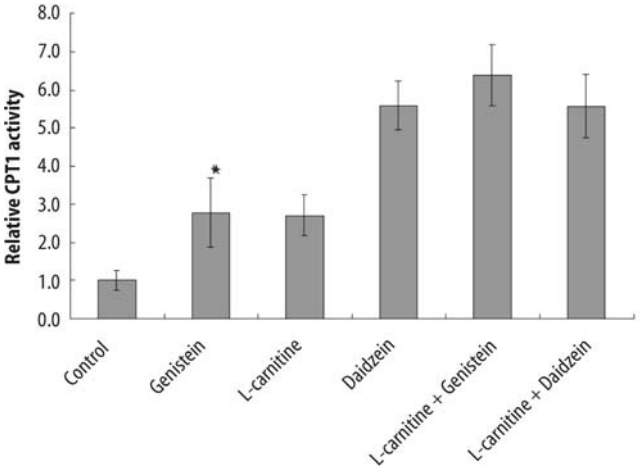

Fig. 1 Effect of genistein (10 μM), daidzein (10 μM) and L-carnitine (1 mM) on CPT1A enzyme activity in HepG2 cells. HepG2 cells were incubated in serum-free media for 24 h and treated with 10 μM genistein or 10 μM daidzein or 1 mM L-carnitine or in combination with 10 μM genistein and 1 mM L-carnitine or in combination with 10 μM daidzein and 1 mM L-carnitine for 48 h. The activity of CPT1A was measured using Ellman's reagent. The bars represent mean of relative CPT1A activity normalized to the activity of the untreated cell (control). Error bar indicates standard deviation (n = 5). * $P < 0.01$ vs. control

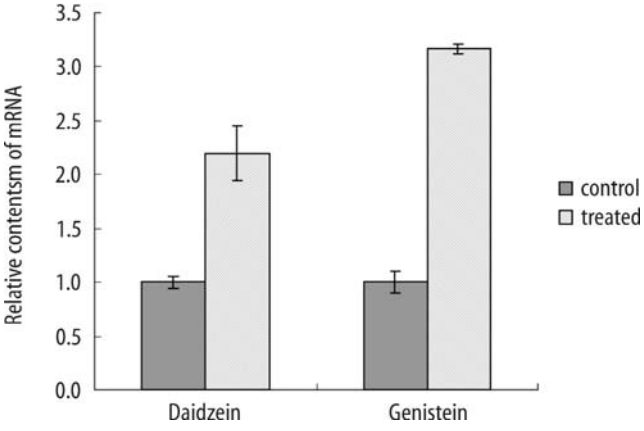

Fig. 2 Change of CPT1A mRNA contents after 10 μM daidzein or 10 μM genistein treatment. HepG2 cells were incubated in serum-free DMEM with 10 μM daidzein or 10 μM genistein for 24 hours. Cells for control group were incubated in serum-free DMEM without daidzein or genistein for 24 hours. The relative contents of mRNA were calculated by using the comparative C_T method. GAPDH mRNA was used as the invariant control. Error bar indicates standard deviations. All measurements were performed in triplicate with three different samples

A CPT1A promoter luciferase reporter gene transfected stable Huh7 cell line

Huh7 cells were transfected with a promoter reporter gene construct, which contains a promoter fragment of the CPT1A 5′-untranslated region from –562 to +1890 relative to the transcription start point (Fig. 3). A stable cell line transfected with the promoter reporter gene

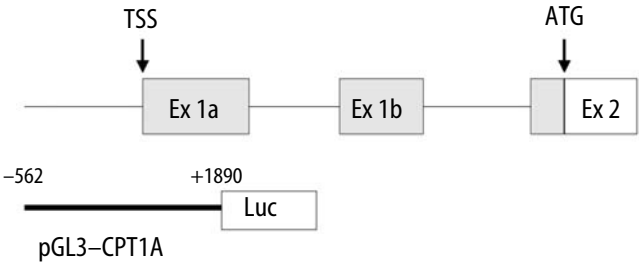

Fig. 3 CPT1A promoter luciferase reporter gene constructs. A promoter fragment of the CPT1A 5′-untranslated region from –562 to +1890 relative to the transcription start site was cloned from human genomic DNA. Promoter fragment was cloned into Kpn I and Bgl II sites of pGL3-Enhancer vector to generate pGL3-CPT1A

construct was selected. To determine whether luciferase activity of the cell line can be regulated by CPT1A promoter fragment, the cell line was cultured in clofibrate containing medium for 24 hours. By 250 μM or 500 μM treatments of clofibrate, luciferase activities were 1.5-fold and 1.8-fold increased, respectively. Luciferase activity of 50 μM or 100 μM clofibrate treated cells were similar to that of control cells (Fig. 4).

To further verify that the luciferase activity of the cell line can be regulated by CPT1A promoter fragment, luciferase activity was measured after the treatment with daidzein or genistein for 24 hours. Treatment with daidzein or genistein increased the luciferase activity significantly (Fig. 5).

The effect of L-carnitine on CPT1A promoter activity

L-carnitine increases CPT1A enzyme activity in HepG2 cells and co-treatment with L-carnitine and genistein additively increases CPT1A enzyme activity in HepG2

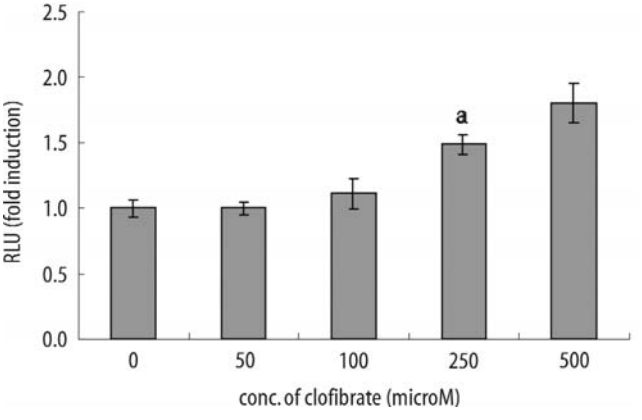

Fig. 4 Effect of clofibrate on CPT1A promoter activity. Transfected Huh7 cells were treated with clofibrate 50 μM or 100 μM or 250 μM or 500 μM for 24 hours. Luciferase activity was measured after treatment with clofibrate. Luciferase activity is presented as X-fold induction relative to the activity measured in untreated cells (control). Error bar indicates standard deviation (n = 3). [a] $P < 0.01$ vs. control

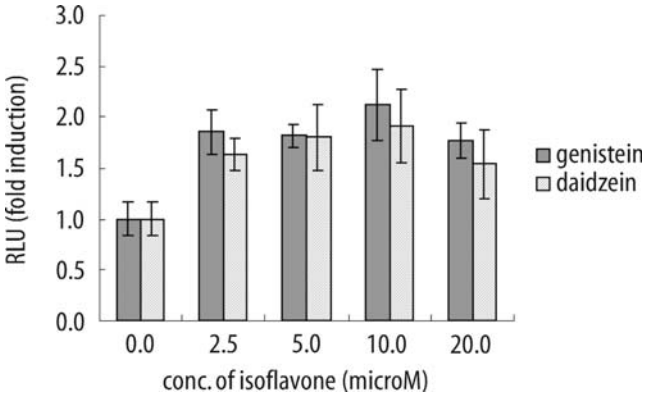

Fig. 5 Effect of genistein and daidzein on CPT1A promoter activity. Transfected Huh7 cells were treated with genistein or daidzein for 24 hours. Luciferase activity is presented as X-fold induction relative to the activity measured in untreated cells (0.0 μM). Solid bars indicate the relative luciferase activity of genistein-treated cells and hatched bars indicate that of daidzein-treated cells. Luciferase activity was measured after 24-hour treatment. Error bar indicates standard deviation (n = 3)

cells (Fig. 1). To determine whether L-carnitine increases CPT1A enzyme activity by directly increasing CPT1A transcription, luciferase activity was measured after the treatment with L-carnitine or in combination with L-carnitine and genistein/daidzein. L-carnitine treatment did not increase luciferase activity. Co-treatment also did not increase luciferase activity more than single-agent treatment with genistein or daidzein (Fig. 6).

Discussion

In regulating β-oxidation pathway, CPT1 enzyme, the outer mitochondrial membrane carnitine palmitoyltransferase, acts as a rate-limiting enzyme [3]. It is also known that the increase in CPT1 mRNA is followed by the increase in CPT1 enzyme activity and consequently results in the increase in fatty acid oxidation and energy expenditure [5]. Many studies have shown that CPT1 gene expression can be regulated by hormonal and nutritional status in cell [2, 5, 6]. CPT1 has at least two isoforms, liver type CPT1 (CPT1A) and muscle type (CPT1B) [6].

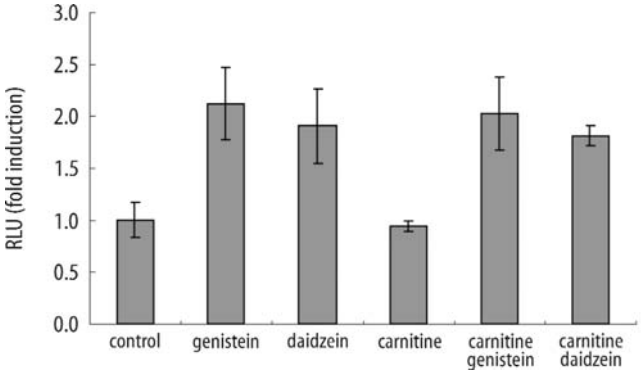

Fig. 6 Effect of daidzein (10 μM), genistein (10 μM) and L-carnitine (1 mM) on CPT1A promoter activity. Transfected Huh7 cells were treated with indicated reagents for 24 hours. Luciferase activity is presented as X-fold induction relative to the activity measured in untreated cells (control). Luciferase activity was measured after 24-hour treatment. Error bar indicates standard deviation (n = 3)

We also found that genistein, one of the major soy isoflavones, increases CPT1A enzyme activity by increasing CPT1A transcription in HepG2 cells [7]. L-carnitine also plays a major role in fatty acid oxidation. L-carnitine levels in hepatocytes modulate hepatic CPT1A activity [12]. In this study, we investigated the effect of co-treatment of genistein and L-carnitine on CPT1A activity in HepG2 cells. The enzyme activity of CPT1A was at least 2.3-fold higher in L-carnitine and genistein co-treated cells than either single-agent treated cells. It can be suggested that co-treatment of genistein and L-carnitine can additively upregulate cellular fatty acid metabolism through upregulation of CPT1A enzyme activity.

Daidzein, another major isoflavone in soy, also increased CPT1A enzyme activity by increasing CPT1A transcription in HepG2 cells. However, daidzein increased CPT1A enzyme activity significantly more than genistein although CPT1A mRNA transcription levels were the opposite. We also investigated the effect of co-treatment of daidzein and L-carnitine on CPT1A activity in HepG2 cells. Unlike co-treatment of genistein and L-carnitine, co-treatment of daidzein and L-carnitine could not additively upregulate CPT1A enzyme activity. These results suggest that other factors could certainly take part in the regulation of CPT1A enzyme activity in HepG2 cells. It may be caused by the different biological activities, such as the differences in tyrosine kinase inhibitory potency and estrogenic effect, of genistein and daidzein. Further studies will be needed to precisely define the molecular mechanisms that generate such differences. Nevertheless, daidzein and genistein increase CPT1A activity, at least in part, by increasing CPT1A mRNA transcription levels and not just by short-term regulation of the activity. These results, in combination with the fact that upregulation of CPT1A is followed by upregulation of fatty acid oxidation, suggest that the soy isoflavones genistein and daidzein can increase mitochondrial β-oxidation by increasing activity of CPT1A, the rate-limiting enzyme in β-oxidation, and subsequently, can increase energy expenditure through enhanced β-oxidation in liver cells.

The organization of the human CPT1A gene was determined and promoter region was suggested [8]. In order to establish a cell line that can be used to screen chemicals to regulate CPT1A transcription, we cloned the CPT1A promoter-luciferase reporter gene construct using the promoter fragment of the CPT1A 5′-untranslated region from −562 to +1890 relative to the tran-

scription start point and a stable cell line was selected after CPT1A promoter-luciferase reporter gene construct was transfected into human hepatoma cell line Huh7. The CPT1A promoter-luciferase reporter gene construct was also transfected into HepG2 cells but we were unable to get a stable cell line. To determine whether the amplified promoter can be regulated by CPT1A up-regulating drug, luciferase activity was measured after clofibrate treatment. Clofibrate is known for peroxisome proliferator and up-regulating CPT1A mRNA levels [2]. Luciferase activity was increased by clofibrate treatment. These results suggest that the amplified human CPT1A promoter region efficiently drives expression of the luciferase reporter gene in the transfected cell line and the amplified promoter region of CPT1A can be regulated by the CPT1 regulating drug.

The target of stimulation by genistein and daidzein is the GC-rich Sp1 binding sequence [13]. In the human CPT1A promoter, three GC-rich Sp1 binding sites have been detected by computer analysis [8]. These GC-rich Sp1 binding sites might be the target of transcriptional regulation by genistein and daidzein. To further verify that the amplified promoter region can drive expression of luciferase reporter gene, genistein and daidzein are treated and the CPT1A promoter luciferase reporter gene transfected in Huh7 cell line. As expected, treatment with genistein or daidzein increased the luciferase activity in the transfected Huh7 cell lines.

L-carnitine upregulates CPT1A enzyme activity in HepG2 cells. However, the promoter activity of CPT1A was not increased by L-carnitine treatment. L-carnitine has the potential to upregulate genes that have TRE (thyroid hormone-responsive element) in their promoter regions [10]. TRE was not detected by computer analysis in the 5′ upstream region of human CPT1A transcription start [8]. No increase in promoter activity by L-carnitine treatment might be caused by the absence of TRE in human CPT1A promoter region.

In summary, we have shown that isoflavones, daidzein and genistein, can up-regulate CPT1A enzyme activity, which is mediated through up-regulation of CPT1A transcription. We also have shown that co-treatment with L-carnitine and genistein additively increases CPT1A enzyme activity in HepG2 cells. In order to establish a cell line that can be used for screening chemicals to regulate CPT1A transcription, a stable cell line was selected after the CPT1A promoter-luciferase reporter gene construct was transfected into the human hepatoma cell line Huh7 and characterized by using clofibrate, genistein and daidzein.

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