Analysis of regulatory mechanisms of an insulin-inducible SHARP-2 gene by (S)-Equol

Ayumi Haneishi a,b, Katsuhito Takagi a, Kosuke Asana a,b, Taichi Yamamotoc, Takashi Tanakac, Soichiro Nakamurab, Tamio Noguchic, Kazuya Yamada a,d,*

a Department of Health and Nutritional Science, Faculty of Human Health Science, Matsumoto University, 2095-1 Niimura, Matsumoto, Nagano 390-1295, Japan
b Laboratory of Molecular Biology, Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nishikiorikita, Tondabayashi, Osaka 584-8540, Japan
c Department of Bioscience and Biotechnology, Shinshu University, 8304 Minamiminowamura, Ina, Nagano 399-4598, Japan
d Department of Health and Nutritional Science, Faculty of Human Health Science, Matsumoto University, 2095-1 Niimura, Matsumoto, Nagano 390-1295, Japan

* Corresponding author at: Matsumoto University Graduate School of Health Science, 2095-1 Niimura, Matsumoto, Nagano 390-1295, Japan. Fax: +81 263 487290.
E-mail address: kazuya.yamada@matsu.ac.jp (K. Yamada).

1 Abbreviations used: SHARP-2, enhancer of split- and hairy-related protein-2; PEPCK, phosphoenolpyruvate carboxykinase; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PVPD, polyvinylidene difluoride; PCR, polymerase chain reaction; m.o.i., multiplicity of infection; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Pi3 K, phosphoinositide 3-kinase; cPKC, classical PKC; nPKC, novel PKC; aPKC, atypical PKC; GFP, green fluorescence protein.

Introduction

Insulin secreted from pancreatic β cell is the only hormone that can lower blood glucose levels. Destruction of pancreatic β cell, relative insulin deficiency, and insulin resistance can result in the onset of the diabetes mellitus [1,2]. Increase incidence of diabetes mellitus is a serious world-health problem and the International Diabetes Federation has predicted the number of diabetic patients will reach over 552 million in 2030 [3]. Therefore, activation of the insulin-signaling pathway in an insulin-independent manner is useful for controlling diabetes mellitus.

The rat enhancer of split- and hairy-related protein-2 (SHARP-2) gene expression may be one of clock-genes in the suprachiasmatic nucleus [4]. SHARP-2 binds to the E box sequence (S-CANNTG) located in the transcriptional regulatory region of several genes [4]. It has been reported that SHARP-2 functions as one of clock-genes in the suprachiasmatic nucleus [5]. We have previously reported that hepatic expression of the rat SHARP-2 gene was induced by feeding a high carbohydrate diet to normal rats or insulin administration to diabetic rats [6,7]. We have also reported that overexpression of SHARP-2 in primary cultured rat hepatocytes and rat H4IIE hepatoma cells decreased the level of phosphoenolpyruvate carboxykinase (PEPCK) mRNA, a gluconeogenic enzyme gene, and that SHARP-2 decreased the promoter activity of the rat PEPCK gene in rat MH1C1 hepatoma cells [8]. Therefore, we hypothesize that SHARP-2 is an important transcription factor involved in the regulation of blood glucose levels [6,8]. Thus, exploration of novel compounds that can increase the SHARP-2 gene expression may be useful for prevention and treatment of diabetes mellitus.

Soybean isoflavones are good candidates since ingestion of them increases insulin sensitivity and decreases the level of fasting plasma glucose, insulin, triglycerides, and total cholesterol in diabetic KKAy mice [9]. In addition, soybean isoflavones can increase insulin secretion and the level of high density lipoprotein-cholesterol and reduce plasma glucose levels in streptozotocin-induced diabetes rats [10]. Molecular species of soybean isoflavones include...
agalloyl glucoside, β-glucoside form, acetyl-glucoside form, and malonyl-glucoside form. In soybean aglycones, there are daidzein, glycitein, and genistein. It has been reported that genistein decreased the level of blood glucose and hemoglobin A1c in diabetic mice and rats and a treatment with genistein promoted the insulin secretion in pancreatic β cells [11–13]. We previously reported that genistein induced the expression of the rat SHARP-2 gene through the activation of protein kinase C (PKC) α [14]. In addition to aglycones, there are β-glucosides, malonyl-glucosides, and acetyl-glucoside configurations [15,16]. The β-glucoside forms are converted to aglycones by β-glucosidase secreted by fermentation microorganisms in soy fermented foods [15,17,18]. Enzymes from saliva and mucosa of small intestine and intestinal bacteria also convert the β-glucoside forms to aglycones [16,19–22]. Daidzein, a soybean isoflavone aglycone, is also metabolized to (S)-Equol by intestinal bacteria [23–26].

In this study, we screened the soybean isoflavones which can induce the rat SHARP-2 gene expression and mainly analyzed a mechanism of induction of the SHARP-2 gene by a metabolite of daidzein, (S)-Equol.

Materials and methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), DMEM (high glucose) without L-glutamine and phenol red, dimethyl sulfoxide (DMSO), 6-O-malonylgenistin, and 6-O-acyetylgenistin were purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS), LY294002, and horseradish peroxidase conjugate-rabbit antimouse IgG antibody were purchased from Sigma Aldrich (Saint Louis, U.S.A.). Streptomyacin and penicillin G were purchased from Meijishiuka (Tokyo, Japan). Genistein, daidzein, glycitein, and genitin were purchased from Fujicco (Kobe, Japan). (S)-Equol was purchased from Cayman Chemical Company (Ann Arbor, U.S.A.). Compound-C, rapamycin, staurosporin, okadaic acid, ICI182,780, cycloheximide, and actinomycin D were purchased from Merck chemicals (Darmstadt, Germany). TRIzol reagent, Lipofectoamine, and Plus reagent were purchased from Invitrogen (Groningen, the Netherlands). High capacity RNA-to-cDNA kit and Big Dye Terminator v1.1 Cycle Sequencing kit were purchased from Applied Biosystems (Foster City, U.S.A.). FastStart Universal SYBR Green Master (Roxy) and Genopure Plasmid Maxi Kit were purchased from Roche Diagnostics (Indianapolis, U.S.A.). The Adeno-X rapid titer kit and rat genomic DNA were purchased from Clontech (Palo Alto, U.S.A.). The Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, U.S.A.).

Preparation of adenovirus

The Ad-GFP and the Ad-dn-αPKCα were generous gifts from Drs. Jun-ichi Miyazaki (Osaka University, Japan) and Wataru Ogawa (Kobe University, Japan), respectively [27,28]. The adenovirus were purified by ultracentrifugation, then followed by dialysis against phosphate-buffered saline [29]. The titration of the adenovirus was carried out using the Adeno-X rapid titer kit. Each adenovirus was infected with the 50 multiplicity of infection (m.o.i.) and cultured for an additional 24 h. The medium was replaced with serum-free DMEM without phenol red supplemented with adenovirus. After two hours, cells were treated with 100 μM (S)-Equol and cultured for another 2 h.

Western blot analysis

H4IE cells were treated with 100 μM (S)-Equol for the indicated times, then harvested in phosphate-buffered saline. The cells were lysed with 12.5 mM Tris–HCl, pH 6.8, 2% glycerol, 1% 2-mercaptoethanol, and 0.5% SDS. The protein concentration was determined using the Bio-Rad Protein Assay. Whole cell lysates were resolved with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a PVDF membrane. Rabbit anti-rat p-PKCα (Ser 657) antibody (SC-12356-R) and rabbit anti-rat PKCα (C-20) antibody (SC-208) were purchased from Santa Cruz Biotechnology (Santa Cruz, U.S.A.). Rabbit anti-rat p-PKCα (Thr 410/403) antibody (9378S) was purchased from Cell Signaling Technology (Danvers, U.S.A.). Mouse anti-rat PKCα antibody (610207) was purchased from BD Biosciences (San Jose, U.S.A.). Horseradish peroxidase conjugate- goat anti-rabbit IgG antibody was purchased from BIOSOURCE (Camarillo, U.S.A.). Hyperfilm ECL was purchased from GE Healthcare (Buckinghamshire, U.K.). The pGL4.20, pGL4.11, pHRluc-CMV plasmids, and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, U.S.A.).
Construction of plasmids

A 3.7 kb Nhe I fragment containing the nucleotide sequences between -3699 and +265 of the rat SHARP-2 gene was subcloned into the Nhe I site of the pGL4.20 to produce pGL4SHARP-2/Luc3700. The pSHARP-2/Luc160 plasmid was previously described [30]. An approximately 200 bp of Kpn I/Hind III fragment of the pSHARP-2/Luc160 was subcloned into the Kpn I/Hind III sites of the pGL4.11 to obtain pGL4SHARP-2/Luc160. Rat genomic DNA was used as the template. PCR was performed in the combination of primers, 5'-CCGGGCTAGCTATCATGCTTACTTTTTAAATA-3' and 5'-CCGGGCTAGTACGCTGCTATTCCCTATATAGC-3', 5'-CCGGGCTAGTCAGTTGCTAGGAG-3' and 5'-CCGGGCTAGTCAGTTGCTAGGAG-3', and 5'-CCGGGCTAGTCAGTTGCTAGGAG-3' and 5'-CCGGGCTAGTCAGTTGCTAGGAG-3', respectively. PCR products were digested with Nhe I, then the approximately 600 bp Nhe I fragments were subcloned into the Nhe I site of the pGL4.11 to give pGL4SHARP-2/Luc160-A, pGL4SHARP-2/Luc-B, and pGL4SHARP-2/Luc-C, respectively.

The nucleotide sequences of all inserts were confirmed using a DNA sequencer 310 Genetic Analyzer (Applied Biosystems).

DNA transfections and luciferase reporter gene assays

All plasmids used for transfection were prepared using the Genopure Plasmid Maxi Kit.

DNA transfections into H4IIE cells were carried out using the calcium phosphate method as described previously [31]. For the analysis of the transcriptional regulatory mechanism(s) of the SHARP-2 gene by (S)-Equol, 10 μg of luciferase reporter plasmid and 0.5 μg of the phRLuc-CMV were co-transfected into H4IIE cells. After transfection, the medium was replaced with serum-free DMEM without phenol red. After 24 h, cells were treated with or without 100 μM (S)-Equol for 2 h.

DNA transfections into MH1C1 cells were carried out using the lipofection method as described previously [32]. Fifty thousands of cells were seeded in a 24 wells plate. After 24 h, cells were transfected with 300 ng of a luciferase reporter plasmid and 0.2 ng of the phRLuc-CMV. After 3 h, the medium was replaced with serum-free DMEM without phenol red. After 24 h, cells were treated with or without 100 μM (S)-Equol for 4 h.

Firefly and sea pansy luciferase assays were carried out using the Dual-Luciferase Reporter Assay System. Procedures were performed according to the manufacturer’s recommended protocol. Luciferase activities were determined on a Berthold Lumat model LB9507 (Wildbad, Germany). Firefly luciferase activities were normalized by sea pansy luciferase activities.

Statistical analysis

All experiments were performed at least three times. Data were represented as the mean and standard error and analyzed by one-way ANOVA, followed by Fisher’s protected LSD multiple comparison test.

Results

Screening of soybean isoflavones to induce SHARP-2 mRNA

In our previous report, genistein, a soybean isoflavone aglycone, induced the expression of the rat SHARP-2 gene in highly-differentiated rat hepatoma H4IIE cells [14]. Soybean isoflavone aglycones and their derivatives are shown in Fig. 1A. Firstly, to examine the effects of soybean isoflavone aglycones on the rat SHARP-2 gene expression, H4IIE cells were treated with 100 μM genistein, daidzein, and glycitein for 2 h. As shown in Fig. 1B, the level of SHARP-2 mRNA significantly increased with genistein and glycitein treatment (P < 0.001). The level of SHARP-2 mRNA induced by genistein was higher than that induced by glycitein (P < 0.001). Next, to examine the effects of various genistin derivatives on the rat SHARP-2 gene expression, H4IIE cells were treated for 2 h with 100 μM genistein, genistin (glucose form of genistein), malonyl-genistin, and acetyl-genistin for 2 h.

Induced expression of SHARP-2 mRNA by (S)-Equol

Daidzein had no effects on the level of SHARP-2 mRNA (Fig. 1B). However, daidzein is metabolized to (S)-Equol by intestinal bacte-
ria in vivo (Fig. 2A) [23–26,33]. Therefore, we then examined whether (S)-Equol induces the rat SHARP-2 gene expression. First, H4IIE cells were treated with various concentrations of (S)-Equol for 2 h. The level of SHARP-2 mRNA increased in a dose-dependent manner (Fig. 2B). Next, the time course for the increase in SHARP-2 mRNA at 100 μM of (S)-Equol was analyzed. The level of SHARP-2 mRNA increased at 1 h, reaching a maximum level at 2 h, and then slightly decreased (Fig. 2C). This time course was similar to those by insulin and genistein as described previously [7,14]. The level of SHARP-2 mRNA by (S)-Equol was higher than that induced by genistein (P < 0.0001).

These results indicate that (S)-Equol is a strong inducer of the SHARP-2 gene expression.

Fig. 2. Effects of (S)-Equol on SHARP-2 mRNA level. The levels of SHARP-2 and 36B4 mRNAs were determined by reverse-transcription and quantitative real-time PCR. Each column and bar represents the mean and standard error of the ratio of the mRNAs were determined by reverse-transcription and quantitative real-time PCR.

Analysis of a signal transduction pathway(s) of an induction of the SHARP-2 mRNA by (S)-Equol

We have previously reported that the induction of SHARP-2 mRNA by insulin and genistein were mediated by the phosphoinositide 3-kinase (PI3 K) and PKC pathways, respectively [7]. We then examined whether the induction of the level of SHARP-2 mRNA by (S)-Equol was mediated by the PI3 K and/or PKC pathways. H4IIE cells were treated with various inhibitors: LY294002 (PI3 K inhibitor), compound-C (AMP-activated protein kinase inhibitor), rapamycin (p70S6 K inhibitor), staurosporin (PKC inhibitor), and okadaic acid (protein phosphatase inhibitor). The induction of SHARP-2 mRNA by (S)-Equol was partially or completely inhibited by a treatment with LY294002 and staurosporin, respectively (Fig. 3).

These results suggest that the induction of SHARP-2 mRNA by (S)-Equol was mediated by both the PI3 K and PKC pathways.

Effects of a dominant negative form of aPKCα on the levels of SHARP-2 mRNA induced by (S)-Equol

PKC has many isoforms such as classical PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) [34]. We previously reported that PKCα, a cPKC, was a mediator of genistein [14]. It has been reported that aPKCα but not PKCα is activated by the PI3 K pathway in the liver [35,36].

We then determined the effect of aPKCα on expression of the rat SHARP-2 gene by (S)-Equol using the adenovirus expression system. The Ad-GFP expresses the green fluorescence protein (GFP), while the Ad-dn-aPKCα expresses a dominant negative form of aPKCα. Expression of GFP and a dominant negative form of aPKCα were confirmed using fluorescence microscopy and Western blot analyses, respectively (data not shown). When the H4IIE cells were infected with the Ad-GFP, the level of SHARP-2 mRNA was not altered (Fig. 4). In contrast, when the Ad-dn-aPKCα was infected to H4IIE cells, the induction level of SHARP-2 mRNA by (S)-Equol was decreased to 56% (P < 0.001).

Activation of PKCα and aPKCα by (S)-Equol

When both PKCα and aPKCα are activated by extracellular stimuli, serine/threonine residues of them are phosphorylated [37]. To
confirm whether PKCα and aPKCα in H4IIE cells were activated by (S)-Equol, whole cell lysates were prepared from (S)-Equol-treated H4IIE cells for Western blot analyses using antibodies against PKCα, phosphorylated PKCα, aPKCα, and phosphorylated aPKCα. An active (phosphorylated) form of PKCα was detected at 5 min post-treatment with (S)-Equol and then decreased by 60 min (Fig. 5). In contrast, the level of whole PKCα protein remained unchanged. An active (phosphorylated) form of aPKCα was detected at 1 min post-treatment with (S)-Equol and this level was maintained until 60 min. In contrast, the level of whole aPKCα protein remained unchanged.

Taken together, these results indicate that (S)-Equol rapidly activates both PKCα and aPKCα.

Estrogen receptor dose not mediate the (S)-Equol effect

(S)-Equol can bind to the estrogen receptor at low binding affinity as well as genistein and it has a weak estrogen activity [24–26,38]. To examine whether the induction of SHARP-2 mRNA by (S)-Equol is mediated by estrogen receptor, H4IIE cells were treated with the estrogen receptor antagonist, ICI182,798. However, the induction level of SHARP-2 mRNA by (S)-Equol was not affected by ICI182,798 (Fig. 6).

This result suggests that the induction of SHARP-2 mRNA by (S)-Equol is not mediated by an estrogen receptor.

(S)-Equol acts at the transcriptional level of the rat SHARP-2 gene

To examine the issue of whether the induction of SHARP-2 mRNA by (S)-Equol is required for de novo RNA and or protein synthesis, H4IIE cells were treated with RNA polymerase II inhibitor (actinomycin D) or protein synthesis inhibitor (cycloheximide). The induction of SHARP-2 mRNA by (S)-Equol was inhibited by both actinomycin D and cycloheximide (Fig. 7).

These results suggest that the induction of SHARP-2 mRNA by (S)-Equol occurred at the transcriptional level of the rat SHARP-2 gene and required for de novo protein synthesis.

(S)-Equol enhances the transcription through the upstream region of the rat SHARP-2 gene

To analyze the mechanism(s) of transcriptional regulation of the rat SHARP-2 gene by (S)-Equol, we used a reporter gene assay...
system. We prepared two reporter plasmids: pGL4SHARP-2/Luc3700 which contains 3.7 kb upstream region from transcription initiation sites of the rat SHARP-2 gene and other is the pGL4.13 which contains both enhancer and promoter regions of the Simian Virus 40 gene. Each reporter plasmid was transfected into H4IIE cells. After 24 h, the cells were cultured in absence or presence of (S)-Equol for 2 h, and then determined their luciferase activities. However, these promoter activities remained unchanged (Fig. 8A).

These results indicate that the 3.7 kb upstream region of the rat SHARP-2 gene does not respond to (S)-Equol.

In order to further explore the (S)-Equol responsive region of the SHARP-2 gene, the following plasmids were prepared: the pGL4SHARP-2/Luc160 contains the nucleotide sequences between −160 and +110 of the rat SHARP-2 gene. This region has been shown to be necessary and sufficient for hepatic expression in MH1C1 cells [30]. DNA fragments containing nucleotide sequences between −4192 to −3760, −4687 and −4133, and −5187 and −4628 were inserted into the pGL4SHARP-2/Luc160 to produce pGL4SHARP-2/Luc160-A, pGL4SHARP-2/Luc160-B, and pGL4SHARP-2/Luc160-C, respectively. Each reporter plasmid was transfected into MH1C1 cells. Only promoter activity of the pGL4SHARP-2/Luc160-B was increased by (S)-Equol (Fig. 8B).

These results indicate that the nucleotide sequences between −4687 and −4133 of the rat SHARP-2 gene contain the (S)-Equol-responsive element(s).

Discussion

Studies of small compounds that stimulates insulin-dependent signaling pathway are useful for prevention and treatment with type 2 diabetes mellitus. We have previously reported that overexpression of SHARP-2 repressed PEPCK gene expression as well as insulin [7]. In the present study, we screened the soybean isoflavone aglycones and their derivatives to determine which compound can induce expression of the insulin-inducible rat SHARP-2 gene. Amongst the soybean isoflavone aglycones and various derivatives of genistein tested, genistein was the strongest inducer for SHARP-2 mRNA expression. We have previously reported that genistein induced the SHARP-2 gene expression via the cPKC pathway [14]. In contrast, daidzein did not affect the induction of SHARP-2 mRNA. Interestingly, (S)-Equol, a metabolite of daidzein, induced the level of SHARP-2 mRNA. It has been reported that approximately 30–50% of the human can produce (S)-Equol from daidzein by normal bacteria flora found in the gut [25,39–42]. When the (S)-Equol producers were compared with non (S)-Equol producers, the levels of serum uric acid and triacylglycerol was low and that of high density lipoprotein–cholesterol was high [43,44]. (S)-Equol rapidly and temporarily induced the expression of SHARP-2 mRNA in H4IIE cells as well as insulin and genistein [7,14]. This induction level was higher than levels of SHARP-2 mRNA induced by genistein. Then, we analyzed the mechanism(s) involved. The PI3 K and cPKC pathways were important for the induction of SHARP-2 mRNA by insulin and genistein, respectively [7,14]. PKC isoforms are classified into cPKC, nPKC, and aPKC [34]. It has been reported that insulin activates aPKCα via PI3 K in the liver [35,36]. As the induction of the SHARP-2 mRNA by (S)-Equol was inhibited by a PI3 K inhibitor, aPKCα was one of the candidate mechanism involved. Indeed, the Ad-dn-aPKCα inhibited the induction of the SHARP-2 mRNA and an active (phosphorylated) form of aPKCα protein was detected in (S)-Equol-treated H4IIE cells.
We have previously reported that phorbol-12-myristate-13-acetate, a cPKC activator, induced the SHARP-2 mRNA in H4IIE cells [14]. The induction of the SHARP-2 mRNA by (S)-Equol was inhibited by staurosporine as well as genistein [14]. Therefore, cPKC was another candidate of (S)-Equol mediator. The cPKC have several isoforms and a major isoform is a PKCα. Phosphorylations of T497, T639, and S657 of cPKCα causes its activation [37]. Indeed, an active (phosphorylated) form of PKCα protein was detected in (S)-Equol-treated H4IIE cells.

(S)-Equol has a weak estrogen activity since chemical structure of (S)-Equol is similar to 17β-estradiol, a female hormone and exhibits low binding affinity to estrogen receptor [24,26,45,46]. The induction of the SHARP-2 mRNA by (S)-Equol was not inhibited by an estrogen receptor antagonist, IC182,780, indicating that a (S)-Equol effect was not mediated by an estrogen receptor. The induction of SHARP-2 mRNA by (S)-Equol was required for de novo RNA and protein synthesis. It has been reported that Equol increases promoter activity of the human cytochrome P450 3A4 gene in HepG2 cells and activates the peroxisome proliferator-activated receptor γ in 3T3-L1 cells [47,48]. Therefore, it is likely that (S)-Equol stimulates transcription of the rat SHARP-2 gene through the activation of some unknown transcription factor(s) located downstream of cPKCα and aPKCα.

A (S)-Equol-responsive region of the SHARP-2 gene was mapped at the nucleotide sequences between −4687 and −4133 of the rat SHARP-2 gene. Although PKC activates some transcription factors, there were no nucleotide sequences bound by these factors [49–51]. In further studies, (S)-Equol-responsive element(s) should be determined.

As summarized in Fig. 9, (S)-Equol is an inducer of expression of the rat SHARP-2 gene, it activates both aPKCα in a PI3K pathway and cPKCα, then either aPKCα or cPKCα or both activate transcription factor Y that stimulates transcription of the transcription factor X gene, and the transcription factor X elevates the promoter activity through the nucleotide sequences between −4687 and −4133 of the rat SHARP-2 gene (Fig. 9).

We have previously reported that the PEPCX gene was one of the SHARP-2-target gene [8]. It raises a possibility that (S)-Equol lowers blood glucose levels via the induction of SHARP-2 and the concomitant repression of PEPCX. Thus, we conclude that (S)-Equol is an useful dietary supplement to control type 2 diabetes mellitus.

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