

Genistein stimulates the insulin-dependent signaling pathway

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Materials
 - 3.2. Cell culture
 - 3.3. Preparation of total RNA and real-time polymerase chain reactions (PCR)
 - 3.4. Western blot analysis
 - 3.5. Statistical analysis
4. Results
 - 4.1. Induced expression of SHARP-2 mRNA by genistein
 - 4.2. Effects of various pathway inhibitors on genistein-induced SHARP-2 mRNA levels
 - 4.3. Activation of PKC α by genistein
 - 4.4. Genistein acts at the transcriptional level of the rat SHARP-2 gene
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

Small compounds that activate the insulin-dependent signaling pathway have potential therapeutic applications in controlling insulin-independent diabetes mellitus. In this study, we investigated whether soybean isoflavones could induce the expression of SHARP-2, a downstream component of insulin-dependent signaling pathway, associated with the regulation of blood glucose. One such compound called genistein, rapidly and temporarily induced SHARP-2 mRNA levels in a dose-dependent manner in rat H4IIE hepatoma cells. This induction process was rapidly stimulated by a protein kinase C (PKC) activator and blocked by a PKC inhibitor, suggesting that SHARP-2 may be induced via PKC activation. Upon Western blot analysis, genistein showed a stimulation of PKC phosphorylation. Therefore, we concluded that genistein might transcriptionally induce SHARP-2 through the activation of PKC in H4IIE cells. Our results suggest that genistein might be a useful dietary supplement to control insulin-independent diabetes mellitus by inducing the SHARP-2 expression via a bypass of the insulin-dependent signaling pathway.

2. INTRODUCTION

The onset of lifestyle diseases is not only caused by the various genetic or epigenetic backgrounds of individuals but also environmental factors such as daily diet intake (1, 2). Indeed, the consumption of high-energy diets such as high carbohydrate and fat causes obesity and insulin resistance, leading to the onset of diabetes mellitus and arterial sclerosis (3-5).

The rat enhancer of split- and hairy-related protein-2 (SHARP-2, also referred to as DEC1, Stra13, and bhlhe40) is a transcriptional repressor that binds to the E-box sequence (5'-CANNTG-3') located in the transcriptional regulatory region of various genes (6). It has been reported that SHARP-2 functions as one of circadian-genes in the suprachiasmatic nucleus (6, 7). The level of hepatic SHARP-2 mRNA increases in normal rats fed with a high carbohydrate diet or in diabetic rats administered with insulin. Overexpression of SHARP-2 in primary rat hepatocytes decreased the levels of gluconeogenic phosphoenolpyruvate carboxykinase (PEPCK) mRNA (8, 9). Therefore, we hypothesized that SHARP-2 is an

Genistein mimics insulin action

important transcription factor involved in the regulation of blood glucose levels (6, 10). Green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), has been shown to rapidly increase SHARP-2 mRNA levels while simultaneously decreasing PEPCK mRNA levels in highly differentiated rat hepatoma H4IIE cells (11-13). We have previously reported the induction of SHARP-2 mRNA by EGCG occurs as rapidly as with insulin (9). Small compounds such as EGCG, might have the capacity to increase SHARP-2 mRNA levels and regulate the blood glucose level. These compounds can be used to improve metabolic diseases such as insulin resistance and diabetes mellitus. It has been reported that the level of blood glucose decreased in diabetic rats fed with a genistein-containing diet (14). Soybean isoflavones such as genistein and daidzein belong to the polyphenol group. These compounds share a similar structure except for the 5-hydroxyl group. As soybean isoflavones are structurally similar to estrogen, a steroid hormone, isoflavones can bind to the estrogen receptors at low-affinity and exhibit weak estrogen activity (15). In addition, soybean isoflavones also have antioxidant effects and other favorable biological activities (16).

In the present study, we evaluated the role of isoflavones on the insulin-dependent signaling pathway using SHARP-2 as a marker gene and showed that genistein induced SHARP-2 mRNA via the activation of the classical protein kinase C (PKC) pathway.

3. MATERIALS AND METHODS

3.1. Materials

Dulbecco's modified Eagle's medium (DMEM), DMEM (high glucose) without L-glutamine and phenol red, and dimethyl sulfoxide (DMSO) were purchased from Wako (Osaka, Japan). Fetal bovine serum and LY294002 were purchased from Sigma Aldrich (Saint Louis, U.S.A.). Streptomycin and penicillin G were purchased from Meijiseika (Tokyo, Japan). Genistein and daidzein were purchased from Fujicco (Kobe, Japan). Compound-C, rapamycin, staurosporin, PD98059, JNK inhibitor II, okadaic acid, phorbol-12-myristate-13-acetate (PMA), actinomycin D, and cycloheximide were purchased from Merck chemicals (Darmstadt, Germany). TRIzol reagent was purchased from Invitrogen (Groningen, the Netherlands). High capacity RNA-to-cDNA kit was purchased from Applied Biosystems (Foster City, U.S.A.). FastStart Universal SYBR Green Master (Rox) and Genopure Plasmid Maxi Kit were purchased from Roche Diagnostics (Indianapolis, U.S.A.). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, U.S.A.). Polyvinylidene difluoride (PVDF) membrane was purchased from MILLIPORE (Bedford, U.S.A.). Rabbit anti-rat p-PKC α (Ser 657) antibody (SC-12356-R) and rabbit anti-rat PKC α (C-20) antibody (SC-208) were purchased from Santa Cruz Biotechnology (Santa Cruz, U.S.A.). Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody was purchased from BIOSOURCE (Camarillo, U.S.A.). Amersham ECL Plus Western Blotting Detection System was purchased from GE Healthcare (Buckinghamshire, U.K.). Bio Max MS Film was purchased from KODAC (Rochester, U.S.A.).

3.2. Cell culture

Rat H4IIE hepatoma cells were a generous gift from Dr. Daryl K. Granner (Vanderbilt University, U.S.A.). Cells were grown in DMEM supplemented with 10 % fetal bovine serum, 100 μ g/ml streptomycin, and 100 units/ml penicillin G at 37 °C in a 5 % CO₂ incubator. One million of cells were seeded in a 6-cm dish. After 24 hours, the medium was replaced with serum-free DMEM without phenol red supplemented with 0.03 % L-glutamine and then cultured for another 24 hours. At two hours after the medium was replaced with the same medium, cells were treated with various concentrations of an isoflavone for various times. To analyze a signal transduction pathway(s), H4IIE cells were pre-treated with various inhibitors for the indicated times. LY294002 (50 μ M), compound-C (1 μ M), rapamycin (0.1 μ M), staurosporin (0.1 μ M), PD98059 (25 μ M), JNK inhibitor II (10 μ M), okadaic acid (10 nM), actinomycin D (0.8 μ M), and cycloheximide (10 μ M) were used as inhibitors. H4IIE cells were also treated with 1 μ M PMA for 2 hours.

3.3. Preparation of total RNA and real-time polymerase chain reactions (PCR)

Total RNA was prepared from cells using the TRIzol reagent. Total RNA (1 μ g) was reverse-transcribed with the High capacity RNA-to-cDNA kit. Procedures were performed according to the manufacturer's recommended protocol. Quantitative real-time PCR was carried out using the cDNA, primers, and FastStart Universal SYBR Green Master (Rox) by means of an ABI 7300 Real Time PCR System. Combinations of SHARP-2 forward 5'-GCGTCAGCACAATTAAGCAAGA-3' and SHARP-2 reverse 5'-GGGTGAGGCCCAAGAAATG-3', and 36B4 forward 5'-GGCGACCTGGAAGTCCAAC-3' and 36B4 reverse 5'-GGATCTGCTGCATCTGCTTG-3', respectively, were used as the primers. Relative amounts of both SHARP-2 and 36B4 mRNA levels were determined. The level of SHARP-2 mRNA was normalized by that of 36B4 mRNA.

3.4. Western blot analysis

H4IIE cells were treated with 100 μ M genistein for the indicated times, then harvested in phosphate-buffered saline. The cells were lysed with 62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 5 % 2-mercaptoethanol, and 2.5 % SDS. The protein concentration was determined using the Bio-Rad Protein Assay. Whole cell lysates (20 μ g/lane) were resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Rabbit anti-rat p-PKC α (Ser 657) antibody (SC-12356-R) or rabbit anti-rat PKC α (C-20) antibody (SC-208) was used as primary antibody. Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody was used as the second antibody. The proteins were visualized using the Amersham ECL Plus Western Blotting System and Bio Max MS Film.

3.5. Statistical analysis

All experiments were performed at least three times. Statistical differences were determined using the two-tailed Student's t-test.

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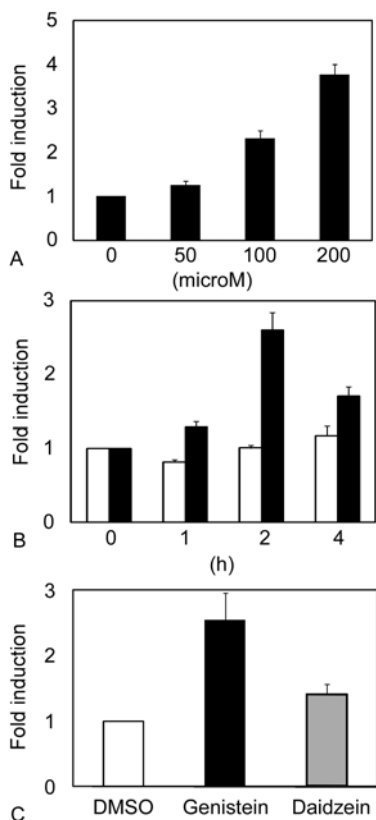


Figure 1. Effects of soybean isoflavones on *SHARP-2* mRNA level. Total RNA was prepared from H4IIE cells treated with soybean isoflavones. The levels of *SHARP-2* and *36B4* mRNA were determined by reverse-transcription and real-time PCR. Each column and error bar represents the mean and standard error of the *SHARP-2* and *36B4* mRNA expression level ratio determined from at least three independent experiments. Expression level ratio in the absence of isoflavones was normalized to one. (A) H4IIE cells were treated for 2 hours with varying concentrations of genistein. (B) Alternations in *SHARP-2* mRNA levels by genistein induction observed after different time points. H4IIE cells were treated with DMSO (white columns) or 100 μ M genistein (black columns) for the indicated time. (C) H4IIE cells were treated for 2 hours with DMSO, 100 μ M genistein or 100 μ M daidzein as indicated.

4. RESULTS

4.1. Induced expression of *SHARP-2* mRNA by genistein

To examine the effects of soybean isoflavones on the expression of the rat *SHARP-2* gene, H4IIE cells were first treated for 2 hours with various concentrations of genistein. The level of *SHARP-2* mRNA increased with genistein in a dose-dependent manner (Figure 1A). *SHARP-2* mRNA was rapidly and temporarily induced using 100 μ M genistein (Figure 1B), which was similar to that by insulin as previously reported (8). Although we also examined the effects of another isoflavone called daidzein, it did not appear to significantly induce the signaling

pathway above background (Figure 1C). These results indicate that genistein specifically induced *SHARP-2* gene expression in H4IIE cells.

4.2. Effects of various pathway inhibitors on genistein-induced *SHARP-2* mRNA levels

To determine the signaling pathway(s) involved in the induction of *SHARP-2* mRNA by genistein, H4IIE cells were treated with various pathway inhibitors. Firstly, to examine whether the induction of *SHARP-2* mRNA by genistein was mediated by the phosphoinositide 3-kinase (PI3K) pathway, H4IIE cells were treated with the PI3K inhibitor LY294002 (8). While the induction of *SHARP-2* mRNA by insulin was inhibited by LY294002, cells induced by genistein were unaffected (Figure 2A). These results indicate that the PI3K pathway was not involved in the induction of *SHARP-2* mRNA by genistein.

Since adiponectin secreted from adipocytes inhibits gluconeogenesis through the AMP-activated protein kinase (AMPK) pathway (17), we examined whether the induction of *SHARP-2* mRNA by genistein was mediated by the AMPK pathway. H4IIE cells treated with the AMPK inhibitor compound-C did not inhibit *SHARP-2* mRNA induction by genistein, indicating that the AMPK signaling pathway was not involved in the genistein-dependent induction of *SHARP-2* mRNA (Figure 2B).

To elucidate other possible genistein-dependent signaling pathway(s) underlying the induction of *SHARP-2* mRNA, H4IIE cells were treated with other inhibitors: Rapamycin (p70S6K inhibitor), staurosporin (PKC inhibitor), PD98059 (MAP kinase inhibitor), JNK inhibitor II (Jun N-terminal kinase inhibitor), and okadaic acid (protein phosphatase inhibitor). Only staurosporin completely inhibited the genistein-dependent induction of *SHARP-2* mRNA, suggesting that *PKC* activation was required in the induction of *SHARP-2* mRNA (Figure 2C). To confirm the involvement of the *PKC* signaling pathway in genistein-mediated induction of *SHARP-2* gene, genistein-treated H4IIE cells were incubated for 2 hours with the *PKC* activator PMA. *SHARP-2* mRNA was greatly induced by PMA, indicating that genistein-dependent induction of *SHARP-2* mRNA was mediated by the *PKC* signaling pathway (Figure 2D).

4.3. Activation of *PKC α* by genistein

To confirm that *PKC* in H4IIE cells was activated by genistein, whole cell lysates were prepared from genistein-treated H4IIE cells for Western blot analysis using antibodies against *PKC α* or phosphorylated *PKC α* . Elevated active-phosphorylated *PKC α* level was detected at 5 minutes post-treatment but this level rapidly decreased by 15 minutes post-treatment with genistein while the level of whole *PKC α* protein remained unchanged (Figure 3). These results suggest that genistein induced *SHARP-2* mRNA by rapidly activating *PKC α* .

4.4. Genistein acts at the transcriptional level of the rat *SHARP-2* gene

We then examined whether the induction of *SHARP-2* mRNA by genistein is required for *de novo* RNA

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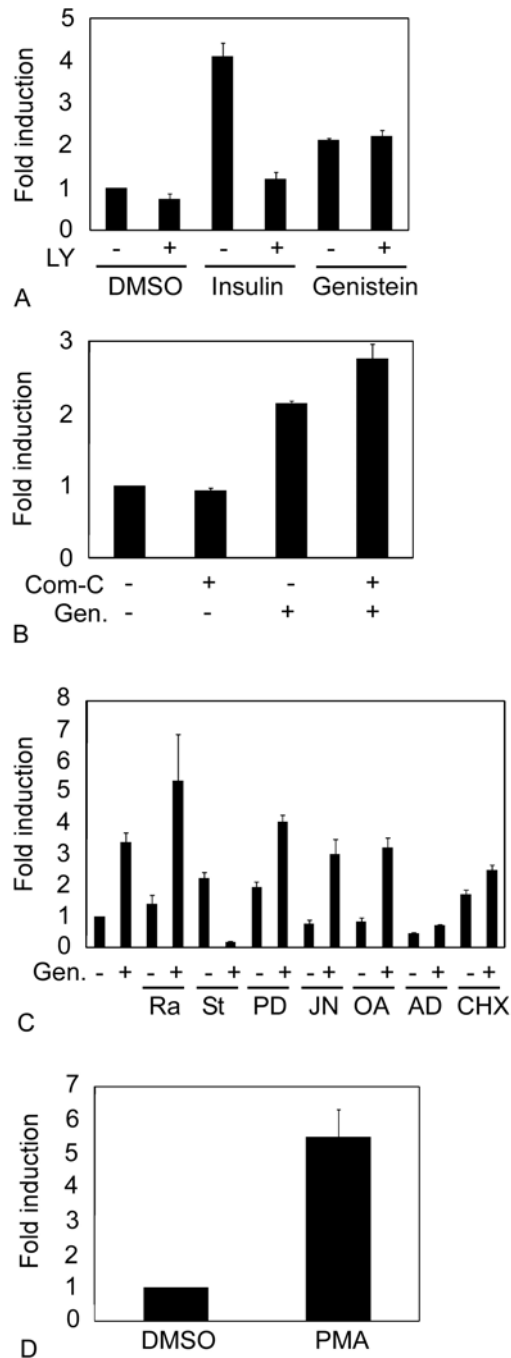


Figure 2. Elucidating the signaling pathway involved in genistein-induced expression of *SHARP-2* mRNA. The levels of *SHARP-2* mRNA in H4IIE cells cultured in various conditions were determined by reverse-transcription and real-time PCR. Each column and error bar represents the mean and standard error of the *SHARP-2* and *36B4* mRNA expression level ratio from four independent experiments. Expression level ratio in the absence of inhibitors (A-C) or PMA (D) was normalized to one. (A) H4IIE cells were initially treated with 50 μ M LY294002 for 30 minutes before treatment with (+) or without (-) DMSO, 10 nM insulin or 100 μ M genistein for another 2 hours. (B) H4IIE cells treated with or without 1 μ M compound-C for 30 minutes were then treated with or without 100 μ M genistein for another 2 hours. (C) H4IIE cells were pretreated for 15 minutes with 0.1 μ M rapamycin (Ra), 0.1 μ M staurosporin (St), 25 μ M PD98059 (PD), 10 μ M JNK inhibitor II (JN), 10 nM okadaic acid (OA), 0.8 μ M actinomycin D (AD), or 10 μ M cycloheximide (CHX) as indicated before being treated with (+) or without (-) 100 μ M genistein for another 2 hours. (D) H4IIE cells were treated for 2 hours with DMSO or 1 μ M PMA.

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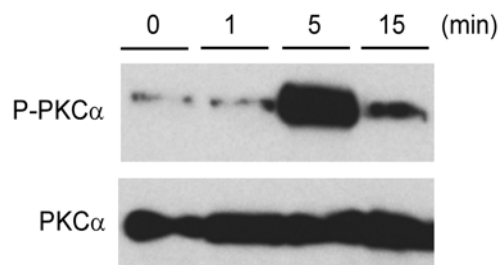


Figure 3. Activation of PKC α by genistein. Whole cell lysates were prepared from H4IIE cells treated with 100 μ M genistein for the times indicated. Whole cell lysates (20 μ g/ lane) were resolved using a 10% SDS-PAGE gel and transferred onto a PVDF membrane for Western blot analysis. The rabbit anti-rat p-PKC α (Ser 657) antibody (1:400 dilution) (*top*) or the rabbit anti-rat PKC α (C-20) antibody (1:400 dilution) (*bottom*) was used as the primary antibody and horseradish peroxidase conjugate-goat anti-rabbit IgG antibody (1:20,000 dilution) was used as the second antibody. Three independent experiments were performed and a representative result shown.

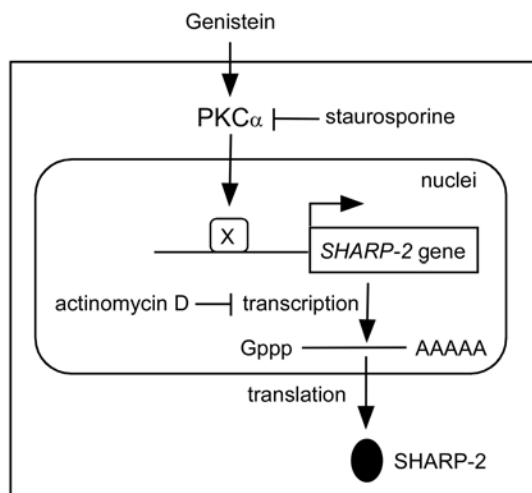


Figure 4. A schematic drawing displaying the potential pathway involved in genistein-induced expression of the rat *SHARP-2* gene.

and/or protein synthesis. H4IIE cells were treated with either a RNA polymerase II inhibitor (actinomycin D) or protein synthesis inhibitor (cycloheximide). The induction of SHARP-2 mRNA by genistein was inhibited by both inhibitors, suggesting that the induction occurred at the transcriptional level and was required for *de novo* protein synthesis (Figure 2C).

5. DISCUSSION

Exploration of small molecular compounds that bypass the insulin receptor and stimulate factor(s) involved in the insulin-dependent signaling pathway may be useful for the prevention of diabetes mellitus, in particular, insulin-resistant diabetes mellitus. We have previously

reported that the insulin-inducible transcription factor SHARP-2 repressed the expression of the rat gluconeogenic enzyme PEPCK gene that elevates the blood glucose level (8, 9). Here, we found that the soybean isoflavone genistein induced SHARP-2 gene expression in H4IIE cells. Like insulin, genistein induced the level of SHARP-2 mRNA in a dose-dependent manner, with a peak expression level attained in 2 hours (Figures 1A and 1B). Daidzein, another soybean isoflavone, had no effect on the level of SHARP-2 mRNA (Figure 1C), indicating that this induction process was specific to genistein. It has been reported that both genistein and daidzein have estrogen-like activity, similar to a phytoestrogen (15). Since daidzein had no effect on SHARP-2 mRNA, it is our hypothesis that genistein regulates SHARP-2 gene expression by another mechanism(s) other than functioning as a phytoestrogen. The level of SHARP-2 mRNA was inducible by insulin within 2 hours and this induction could be inhibited by the PI3K inhibitor LY294002 (8). Adiponectin secreted from adipocytes inhibits gluconeogenesis through a AMPK pathway (17). However, the induction of SHARP-2 mRNA by genistein was not affected by these inhibitors (Figures 2A and 2B). These results revealed that both PI3K and AMPK pathways, known insulin-dependent signaling pathways, did not mediate the induction of SHARP-2 mRNA. Therefore, we attempted to identify the signaling pathway(s) involved in genistein-induced SHARP-2 gene expression by using the various pathway inhibitors. Surprisingly, genistein-induced SHARP-2 gene expression was inhibited by the PKC inhibitor staurosporin (Figure 2C). PKC has many isoforms and the atypical PKC λ (aPKC λ) isoform has been reported to be activated by the PI3K pathway in the liver (18). The induction of SHARP-2 mRNA by genistein was not inhibited by LY294002, indicating that aPKC λ was not involved in this induction. In fact, when H4IIE cells were treated with PMA that activates classical PKC (cPKC) but not aPKC λ , SHARP-2 mRNA was induced by 2 hours (Figure 2D). These results revealed that cPKC was involved in genistein-induced SHARP-2 gene expression. Because phosphorylated PKC α , a cPKC isoform, is an activated form (18), we examined whether the PKC α was phosphorylated by genistein. Elevated levels of active phosphorylated form of PKC α were detected at 5 to 15 minutes in H4IIE cells post-treated with genistein (Figure 3). It has also been reported that the phosphorylated form of PKC α was detectable at 24 hours in human hepatoma HepG2 cells post-treated with genistein (19). While our results showed that PKC α was rapidly and temporarily activated in H4IIE cells treated with genistein, this difference in temporal induction could be due to varying experimental and growth culture conditions including the cell lines used.

Genistein-induced SHARP-2 gene expression was inhibited by actinomycin D, suggesting that this induction was controlled at the transcriptional level (Figure 2C). It has been reported that genistein increased promoter activity of the *apolipoprotein A-I* gene in HepG2 cells and the expression of peroxisome proliferator-activated receptor transcription factor was also induced by genistein (20, 21). Therefore, it is likely that genistein increased the transcription rate of the rat SHARP-2 gene through the

Genistein mimics insulin action

activation of some unknown transcription factor(s) located downstream of PKC α (Figure 4). To identify the genistein-responsive *cis*-acting element(s) of the SHARP-2 gene, a 3-kb region upstream from the transcription initiation site of the rat SHARP-2 gene was examined in preliminary experiments. Although this region contains multiple elements that could mediate transactivation by various stimulations, genistein treatment could not stimulate promoter activity in H4IIE cells transfected with the luciferase reporter plasmid containing the 3-kb promoter region (data not shown) (6).

We conclude that genistein induces expression of the rat SHARP-2 gene through the activation of PKC α and could potentially be used as a dietary supplement to control insulin-independent diabetes mellitus. Further studies are required for the identification of the elusive genistein-responsive element of the rat SHARP-2 gene in order to fully understand its transcriptional regulatory mechanism(s).

6. ACKNOWLEDGEMENTS

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Genistein mimics insulin action

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Abbreviations: SHARP-2, enhancer of split- and hairy-related protein-2; PEPCK, phosphoenolpyruvate carboxykinase; EGCG, (-)-epigallocatechin-3-gallate; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; PMA, phorbol-12-myristate-13-acetate; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PI 3-K, phosphoinositide 3- kinase; AMPK, AMP-activated protein kinase; aPKC λ , atypical PKC λ ; cPKC, classical PKC

Key Words: Soybean Isoflavones, Signaling Pathway, Gene Expression, Transcription, Classical Protein Kinase C

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