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Genistein and daidzein induced apoA-1 transactivation in hepG2 cells expressing oestrogen receptor-α

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Studies have shown that soya consumption has been associated with low incidence of CVD. Because the chemical structures of soya isoflavones are similar to oestrogen, the beneficial outcome may be attributed to the oestrogenicity of these compounds. In this study, effect of the soya isoflavone genistein on the mRNA expression of apoA-1 in the human hepatoma HepG2 cell was investigated. Without oestrogen receptor (ER) α transfection, soya isoflavones in the physiological range had no effect on the apoA-1 transcription. Once ERα was ectopically expressed in these cells, soya isoflavone dramatically increased the apoA-1 mRNA abundance quantified by real-time PCR. *ApoA-1*-reporter assays with plasmid constructed from the 5′-flanking segment upstream to the coding region revealed that the transactivation of the *apoA-1* promoter was induced by the soya isoflavone in HepG2 cells expressing ERα. This induction was reduced by the anti-oestrogen ICI 182780, but not the inhibitors of protein kinase (PK) C, PKA, or mitogen-activated PK. Based on the previously identified response elements on the promoter, a series of truncated promoter reporter plasmids were then constructed. An induction profile of genistein was built and insulin response core element at −411 to −404 appeared to be a potential site of interaction. This study illustrated that soya isoflavones at physiological concentrations could up regulate apoA-1 mRNA expression in ERα-transfected HepG2 cells.

Genistein: Oestrogen receptor: ApoA-1: Liver cancer cells

CVD comprise the major cause of death in Western countries. Recent projections suggest that CVD will be the leading cause of death in both developed and developing regions of the world by the year 2020[1]. Epidemiological studies have associated the consumption of isoflavonoids with a lower incidence of CVD[2]. In normal postmenopausal women, consuming whole soya foods with 60 mg isoflavones per d may help alleviate several key clinical risk factors for CVD[3].

HDL is synthesised in hepatic and intestinal cells and secreted as small particles containing phospholipids, free cholesterol, apoA-1 and apoE. Cholesterol synthesised or deposited in peripheral tissues is returned to the liver in a process referred to as 'reverse cholesterol transport'. *ApoA-1* activates lecithin–cholesterol acyltransferase and facilitates the removal of cholesterol from the tissues (as reviewed by Fielding & Fielding[4]). *ApoA-1* is a major constituent of HDL at the structural as well as the functional levels. The promoter region of the human *ApoA-1* gene has been characterised to be tissue-specific. In the liver, the transcription begins at 235 base pairs upstream to the translation start site[5]. Several *cis* enhancer or suppressor DNA-binding elements responding to changes in hormonal or metabolic status have been identified. An insulin response core element (IRCE) situated at −411 to −404 is responsible for insulin-[6] and epidermal growth factor-[7] induced gene expression. In the span from −214 to −119, three crucial elements: site A (−214 to −192), site B (−169 to −146) and site C (−134 to −119) have been described for the binding of transcriptional factors and nuclear receptors[8].

ERα is a member of nuclear hormone receptors which bind a wide range of hydrophobic molecules, such as steroid hormone and phyto-oestrogens. ERα is found in various tissues, including liver, bone, heart and central nervous system[9]. Oestrogen binds to the C-terminal domain of ERα in the cytoplasm and releases the heat shock proteins. The activated ERα is translocated into the nucleus and seeks out genes with a specific response element for binding. The gene transcription machinery is then activated and the encoded mRNA is expressed.

Isoflavones are major phytooestrogens that have been the focus of many studies regarding their health benefits. Isoflavones share some common structure with the hormone oestrogen. Despite the similarity, the relative binding affinity of isoflavones for ERα is only 0·05–1 % of the binding affinity of 17β-oestradiol[10]. In contrast, their binding affinity for ERβ is approximately seven-fold greater than that of oestrogen. It is suggested that isoflavones may act as selective ER modulators[11]. In addition, the plasma isoflavone concentration can be several thousand times greater than that of oestradiol[12]. They may compete for ER and display anti-oestrogenic effect, especially when endogenous oestrogen level is low.

Abbreviations: ER, oestrogen receptor; IRCE, insulin response core element; PK, protein kinase; MAPK, mitogen-activated PK.

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Previous studies have shown that human hepatoma HepG2 cells can be a viable model for apo research[13–15] except that these cells do not express ERα[16]. By using this cell model, this study was designed to investigate the regulatory mechanism of soya isoflavone on *ApoA-1*.

Materials and methods

Chemicals

Soya isoflavones were purchased from Sigma Chemicals (St Louis, MO, USA). PD98059, bisindolylmaleimide I, and 14–22 amide were obtained from EMD Biosciences Inc. (La Jolla, CA, USA). All other chemicals, if not stated, were acquired from Sigma Chemicals.

Cell culture

HepG2 cells (American Tissue Culture Collection, Rockville, MD, USA) were routinely cultured in RPMI–1640 media (Sigma Chemicals), supplemented with 10 % fetal bovine serum (Invitrogen Life Technology, Rockville, MD, USA) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin), and incubated at 37°C and 5 % CO_2. Three days before the experiment, the cultures were switched to RPMI–1640 phenol-red-free media (Sigma Chemicals) and 5 % charcoal–dextran-treated fetal bovine serum (Hyclone, Utah, USA). Sub-confluent cell cultures were treated with isoflavone with dimethylsulphoxide as the carrier solvent. The final concentration of the solvent was 0·1 % (v/v), and the control cultures received dimethylsulphoxide only.

Luciferase reporter gene assay

Construction of ApoA-1 activated luciferase reporter plasmids. Fragments from human *ApoA-1* 5′-flanking region were amplified from genomic DNA isolated from HepG2 cells. Primers were designed with the incorporation of Mlu I and Bgl II restriction sites. The forward primers for the respective constructs are listed in Fig. 1. They all shared the common reverse primer, 5′-ACA AGA TCT TTA GGG GAC ACC TAC CCG TCA-3′. The amplified product was then digested and subcloned into a firefly luciferase reporter vector pTA–Luc (BD Biosciences Clontech, Palo Alto, CA, USA), and the sequence accuracy was verified.

Dual luciferase assays. HepG2 cells were seeded at 10^5 cells/well in 24-well plates. After 24 h, the cells were transiently transfected with the reporter and ER expression plasmid at 0·4 μg each and 0·1 μg renilla luciferase control vector pRL (Promega, Madison, WI, USA) in LipofectAmine (Invitrogen Life Technologies). ERα and ERβ expression plasmids were generous gifts from Dr Donald Macdonald of Duke University, Northern Carolina. After 16 h, the medium was removed and the cells were treated with isoflavone for 24 h. The amounts of these two luciferases were determined using Dual-Luciferase Assay Kit (Promega). The luciferase bioluminescence was quantified by using a FLUOstar Galaxy plate reader. The transactivation activities of the *ApoA-1* promoter represented by firefly luciferase light units were then normalised with that of renilla luciferase.

Quantitative real-time PCR assay

HepG2 cells were seeded in a six-well plate for 1 d and transfected with ERα expression plasmid or the empty vector pcDNA3.1. The medium was removed and cells were cultured with soya isoflavone. After 24 h of treatment, total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined by absorbance at 260/280 nM. First DNA strands were synthesised from 3 mg total RNA using oligo-dT primers and M-MLV RT (USB Corporation, Cleveland, Ohio, USA). Target fragments were quantified by real-time PCR, and a DNA Engine Opticon™2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was employed for this assay.

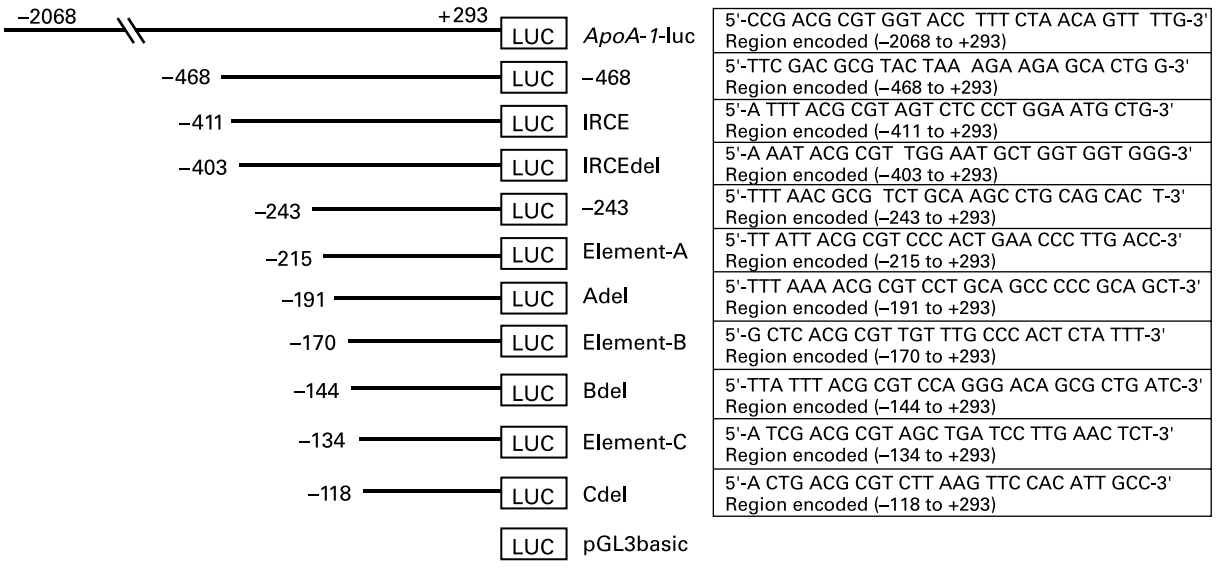

Fig. 1. Construction of the truncated apoA-1 promoter reporter plasmids. IRCE, insulin response core element; del, deletion; luc, luciferase.

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FAM™ dye-labelled TaqMan® MGB probes and primers for apoA-1 and glyceraldehyde-3-phosphate dehydrogenase (Assay-on-Demand™) and real-time PCR Taqman Universal PCR Master Mix were all obtained from Applied Biosystems (Foster City, CA, USA). PCR reactions were set up as described in the protocol, which was validated by the company. Signals obtained for glyceraldehyde-3-phosphate dehydrogenase was used as a reference housekeeping gene to normalise the amount of total RNA amplified in each reaction. Relative gene expression data were analysed using the 2-ΔΔCT method[17].

Electrophoretic mobility shift assay

Nuclear protein extract was isolated by using NucBuster™ protein extraction kit (Novagen®; EMD Biosciences, Inc., La Jolla, CA, USA.). In brief, cells were washed, trypsinised, and packed at 500 ***g*** at 4°C. Reagent 1 was added to the packed cells. Nuclear extract was isolated from the cell suspension by vortexing and centrifugation. The nuclear protein was stored at −80°C until assayed.

Because the deletion of IRCE consistently reduced the transactivation in 0·5 μM- and 10 μM-genistein as shown later in Fig. 4 (B), we pursued the binding activity with electrophoretic mobility shift assay. The annealed oligonucleotide of 2 × IRCE was labelled with T4 polynucleotide kinase (Novagen), and (γ32P)-ATP (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA). A spin column was used to purify the labelled probe. The nuclear protein was incubated with the labelled probe, sonicated salmon sperm DNA, poly(dI-dC), and binding buffer (400 mM-KCl, 80 mM-HEPES, 2 mM-dithiothreitol, 0·8 mM-EDTA, 80 % glycerol, pH 8) provided in electrophoretic mobility shift assay accessory kit (Novagen) for 30 min at room temperature. The reaction mix was then separated by a 6 % non-denaturing gel in 0·5 × Tris-borate EDTA at 100 V. The gel was dried and autoradiographed following the procedures in using a molecular imager FX (BioRad).

Statistical methods

A Prism® 3.0 software package (GraphPad Software, Inc., CA, USA) was utilised for statistical analysis. Results of this study were compared by ANOVA and Bonferroni's method for multiple comparisons. Significance level was set at $P<0·05$.

Results

Effect of soya isoflavone on apoA-1 mRNA expression

Cultures transfected with ERα had a dose-dependent increase in apoA-1 mRNA abundance upon soya isoflavone treatment, whereas the apoA-1 expression in cells transfected with empty vector was not affected by the same treatment (Fig. 2). Isoflavone ranged from 0·1 μM to 1 μM could induce 2·5 to 20-fold increase in mRNA abundance, whereas oestradiol (E2) at 1 nM elicited about 7-fold increase. The inductions at 0·1, 0·5 and 1 μM-daidzein treatment appeared to be stronger, no difference and weaker, respectively, than their genistein counterparts at the same concentrations. No induction was observed in empty vector transfected cultures,

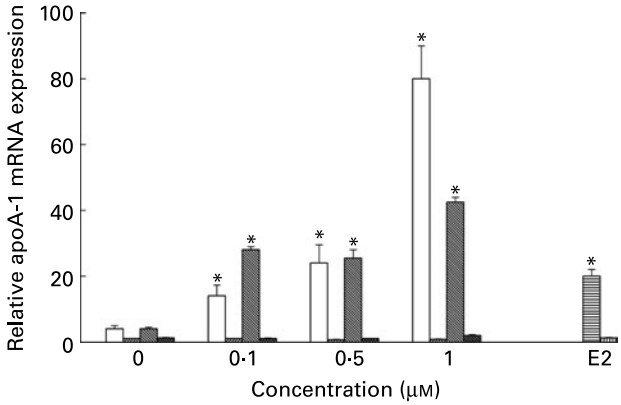

Fig. 2. Effect of soya isoflavone on apoA-1 mRNA expression in the presence (+) and absence (−) of oestrogen receptor (ER)α in HepG2 cells. HepG2 cells were transfected with ERα expression plasmid or pcDNA3.1 vector. After 1 d, cells were treated with soya isoflavone or 1 nM-oestradiol (E2). Total RNA was isolated and the apoA-1 expression was measured. Values are means (n 3) with standard errors indicated by vertical bars for □, genistein (ER +); ▨, genistein (ER −); ▧, daidzein (ER +), ▩, daidzein (ER−); ▤, E2 (ER+); ▥, E2 (ER−). Mean values show a significant increase in expression when compared with the control: *$P<0·05$.

and cultures transfected with ERα had a 3-fold higher apoA-1 mRNA expression than those transfected with control vector.

Response of ApoA-1 promoter to soya isoflavone in HepG2 cells expressing ERα

Following the real-time PCR experiment, we carried out reporter gene assays to verify the expression regulation. Significant elevations in the *ApoA-1* promoter driven luciferase activity were demonstrated in ER-transfected cells treated with soya isoflavone at 0·5 μM or above (Fig. 3), and the increased activities ranging from 400 to 600 % were observed. Oestradiol at 1 nM also induced 6-fold increase in the normalised luciferase activities compared to the control.

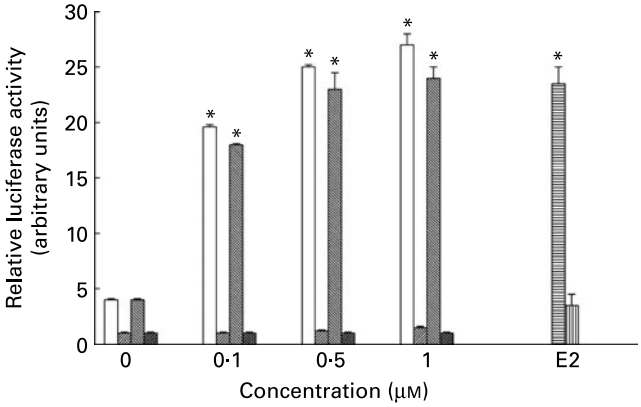

Fig. 3. Soya isoflavone increased *ApoA-1* promoter transactivation in HepG2 cells expressing oestrogen receptor (ER)α. HepG2 cells were plated and transfected with pTA-*ApoA-1*-luciferase, ERα expression plasmid and the control plasmid pRL. Cells were treated with soya isoflavone or oestradiol (E2) for 24 h. Cell extracts were analysed for luciferase activity. Values are means (n 3) with standard errors indicated by vertical bars for □, genistein (ER +); ▨, genistein (ER −); ▧, daidzein (ER +), ▩, daidzein (ER−); ▤, E2 (ER +); ▥, E2 (ER−). Mean values show a significant increase in expression when compared with the control: *$P<0·05$.

Effect of protein kinase inhibitors and antioestrogen on ApoA-1 promoter driven luciferase activities in cultures expressing ERα

Previous studies[18,19] have indicated the involvement of protein kinase (PK) A, PKC or the mitogen-activated protein kinase (MAPK) in *ApoA-1* regulation. Bisindolylmaleimide I, 14-22 amide, and PD98059 and are specific inhibitors for PKC, PKA, and MAPK, respectively. HepG2 cells were transfected with ERα for 1 d, and were then pre-treated with 1 μM-ICI 182, 780, 10 μM-PD 98 059, 10 μM-myristoylated PKI 14-22 amide, or 1 μM-bisindoylmaleimde I. Isoflavone or oestradiol was administered afterwards for 24 h. Compared to the control the administration of these inhibitors did not substantially decrease the *ApoA-1* promoter-driven gene transactivation (Fig. 4). On the contrary, the MAPK and PKC inhibitors significantly increased the promoter activity. When the pure antioestrogen ICI 182730 was administered, the luciferase activity was reduced ($P<0{\cdot}05$) as demonstrated in Fig. 4. These data illustrated that ERα was involved in soya isoflavone-induced *ApoA-1* transcriptional activation.

ApoA-1 promoter analysis in ER-positive HepG2 cells under soya isoflavone treatment

HepG2 cells were transfected with various *ApoA-1* reporter constructs and ERα expression plasmid. Luciferase activity was subsequently measured to reveal the transcriptional control of apoA-1 expression. All *ApoA-1* constructs displayed increasing trends when treated with increased concentrations of genistein (Fig. 5 (A)). In terms of the induced percentage, significant differences were observed between the IRCE and IRCE deletion onstructs, and the element-C and Cdel constructs at 0·5 μM- and/or 10 μM-genistein treatment. Under 10 μM-genistein treatment, only the signal given off by the IRCE stayed higher than the deletion construct. This indicated that IRCE was consistently activated by the soya isoflavone (Fig. 5 (B)).

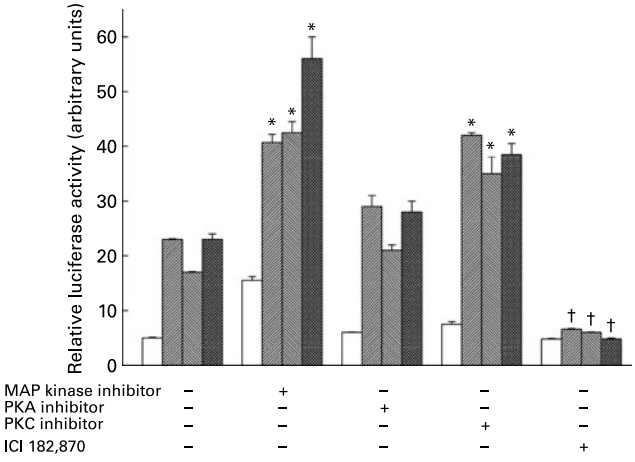

Fig. 4. ICI 182, 780 antagonised soya isoflavone-induced *ApoA-1* promoter transactivity in HepG2 cells expressing oestrogen receptor (ER)α. Cells were transfected with pTA-*ApoA-1*-lucifease plasmid and ERα expression plasmid and then grown for 24 h. The cells were then treated with soya isoflavone or 1 nM oestradiol for 24 h with (+) or without (−) 1 h pretreatment of 1 μM-ICI 182780, 10 μM-PD 98 059, 10 μM-myristoylated PKI 14-22 amide, or 1 μM-bisindoylmaleimide I. Values are means (n 3) with standard errors indicated by vertical bars for □, dimethylsulphoxide; ▨, genistein; ▧, daidzein; ▩, oestradiol. Activity was significantly ($P<0{\cdot}05$) increased (*) or decreased (†) when compared with the control cultures.

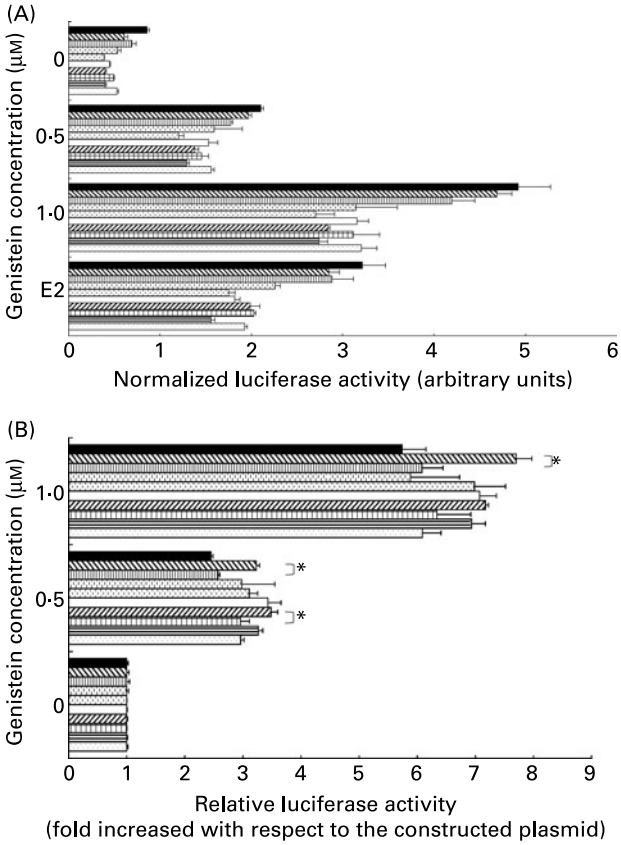

Fig. 5. Genistein induced *ApoA-1* promoter activity profile. HepG2 cells were seeded in 24-well culture plates and transfected with the serial truncation plasmid, oestrogen receptor (ER)α expression plasmid, and renilla luciferase plasmid. After 24 h of transfection, the cultures were treated with genistein for each construct. The cells were lysed and assayed for firefly and renilla luciferase activities. (A) shows one set of two experiments performed with comparable results for ■, −468apoA-1; ▨, insulin response core element (IRCE); ▤, IRCEdel; ⊡, −243apoA-1; ⊡, element A; □ Adel; ▧, element B; ⊞, Bdel; ▥, element C; ⊡ Cdel. Values are means with standard errors indicated by horixontal bars. Mean values show a significant increase in activity when compared with the control cultures: *$P<0{\cdot}05$. (B) is a replot of the values for each of the truncated *ApoA-1* promoter normalised with its own construct without genistein treatment. Values are means with standard errors indicated by horixontal bars, n 3.

DNA binding at IRCE under soya isoflavone treatment

The binding of nuclear protein isolated from ER-positive HepG2 cells to IRCE appeared to be inversely proportional to the concentration of genistein and daidzein (Fig. 6). At 10 μM- genistein or daidzein, the binding to this region was weakened as reflected by the optical density of the retarding band. Oestradiol at 1 nM also displayed a weaker band than the dimethylsulphoxide control (0·1 %). This result suggested that the isoflavones released the transcription suppression rather than encouraging the DNA binding at IRCE.

Discussion

In the present study, we found that soya isoflavone up regulated apoA-1 mRNA expression in HepG2 cells expressing

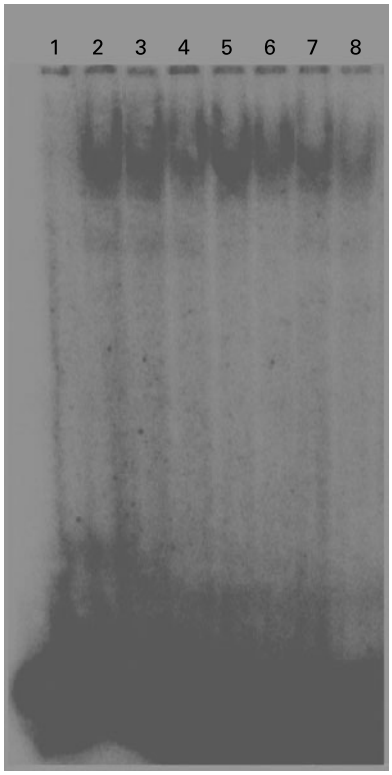

Fig. 6. Insulin response core element (IRCE) binding activity in oestrogen receptor (ER)-positive HepG2 cells under soya isoflavone treatment. HepG2 cells were seeded in 100 mm culture dishes and transfected with ERα expression plasmid. After 24 h of transfection, the cultures were treated with isoflavone for 1 d. Nuclear protein was extracted from the cells and assayed for IRCE binding activities. Lane arrangement: 1, labelled probe only; 2, 0 μM-genistein (0·1 % dimethylsulphoxide); 3, 0·5 μM-genistein; 4, 10 μM-genistein; 5, 0·5 μM-daidzein; 6, 10 μM-daidzein; 7, 1 nM-oestradiol; 8, 100 × cold probe competition.

ERα. *ApoA-1* promoter-driven reporter gene assays supported evidence that the up regulation was introduced by increased transcriptional activities. The induction pathway appeared to be independent of MAPK, PKA, and PKC. Luciferase assays using the truncation reporter plasmids revealed the ICRE lying between −412 and −404 in the 5′flanking region of the *ApoA-1* promoter could be important in the upregulation of transcription by genistein. Besides, genistein appeared to be a stronger inducer of apoA-1 expression than daidzein.

Genistein and 17β-oestradiol have been shown to increase the promoter activities of *ApoA-1* in HepG2 cells[20,21]. Similarly, the present study demonstrated that soya isoflavone activated both the apoA-1 mRNA expression and *ApoA-1* promoter activity. The phytooestrogen soya isoflavone appeared to activate ERα for the induction of mRNA expression of apoA-1, and the condition has not been established in the studies mentioned earlier. It has been shown that a MAPK activation pathway is increased in the up regulation of *ApoA-1*-gene expression by genistein and 17β-oestradiol in wild type HepG2 cells[18]. Conversely, it has been shown that overexpressing MAPK1/2 suppresses rather than promotes the transcriptional activities[18]. Our study indicated that inhibition of several signalling pathways including MAPK, PKC and PKA pathways did not abolish the augmented *ApoA-1* transcription, which is consistent with the latter study. This suggested that the up regulation of *ApoA-1* transcriptional activity in the presence of ERα was not going through these signalling pathways. Some of the kinase inhibitors appeared to stimulate apoA-1 transactivation. The underlying mechanisms warrant further investigation.

Previous studies performed on the wild-type HepG2 cells have shown that oestrogen and genistein increase luciferase reporter activities of *ApoA-1*, and the increase in *ApoA-1* gene promoter transactivities is mediated through the −256 to −41 region of the gene[20,21]. This region contains binding sites for the transcription factors HNF-3β, HNF-4, and Egr-1. Sites at −214 to −192 and −169 to −146 have been shown containing response elements for HNF-4[22] and HNF-3β[23], respectively. Binding sites for Egr-1 have also been located at −221 to −231 and −189 to −181[24]. However, the mRNA expressions in these studies have not been presented. In the present study, neither oestrogen nor soya isoflavone increased apoA-1 mRNA expression in HepG2 cells without ERα. It appears that the increased transactivities reported in these studies fail to bring forth a change at the mRNA level. On the other hand, cells transfected with ERα displayed a stronger increase in mRNA expression than the control even at the base level (0 μM). This increase of apoA-1 mRNA abundance was magnified when soya isoflavone or oestrogen was administered. Genistein or daidzein at 0·1 μM could significantly increase the mRNA amount in ER-positive HepG2 cells.

With respect to our truncation reporter experiments performed in HepG2 cells expressing ERα, deletion of IRCE significantly differentiates the transactivation activity under the treatment of 0·5 μM and 10 μM- genistein. This DNA binding site might be involved in the activation of *ApoA-1* promoter activity. Many transcription factors interacting with insulin response element have been reported, such as Sp1[6], IRE-ABP[25], FOXO1a, FOXO3a, FOXO4a[26], etc. Upon the binding to the insulin-sensitive DNA motif, some factors are activating to the transcription while the others are suppressive. In the present study, our electrophoretic mobility shift assay result suggested that the IRCE binding could be a blockage rather than inductive in the context of soya isoflavone-induced, *ApoA-1*-driven transactivity. Since the difference in transactivity with and without IRCE was about 20 % as shown in the 10 μM-genistein treatment shown in Fig. 4 (B), IRCE could be a part of the binding regions of several co-activators. The binding protein could also be unrelated to apoA-1 transactivation. Considering that the segment between −41 and −256 is reported to be sufficient and specific for maximal *ApoA-1* transcription in HepG2 cells[27], further investigation is required to pinpoint the exact transcriptional mechanism.

Oestrogen replacement therapy has long been used for controlling postmenopausal symptoms, including lowering blood cholesterol. Lamon-Fava *et al.*[20] have demonstrated that oestradiol increases promoter activities of *ApoA-1* in HepG2 cells. An ER-independent pathway has also been described[28] for equine oestrogen in the up regulation of *ApoA-1* promoter activity. In contrast, oestrogen may repress apoA-1 expression. Harnish *et al.*[16] observed that 100 nM- oestradiol represses *ApoA-1* promoter activity in HepG2 cells stably expressing ERα. These contradictory observations can be explained by differences in the oestrogen concentration, timing, or the model nature.

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In summary, the present study demonstrated that soya isoflavone at physiologically relevant concentrations can up regulate *ApoA-1* transcription in HepG2 cells transfected with ERα, but not in the wild-type cells.

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