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Oestrogen receptor α is required for biochanin A-induced apolipoprotein A-1 mRNA expression in HepG2 cells

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Epidemiological studies have indicated that soya consumption may produce a better plasma lipid profile. The effect may be attributed to the phyto-oestrogens in soya. The red clover (*Trifolium pratense*) isoflavone biochanin A has a chemical structure similar to those phyto-oestrogens found in soya beans, and is marketed as a nutraceutical for alleviating postmenopausal symptoms. In the present study we investigated the effect of biochanin A on the mRNA expression of ApoA-1 in the hepatic cell line HepG2. Real-time PCR revealed that biochanin A increased ApoA-1 mRNA abundance in cells expressing oestrogen receptor (ER) α. Without ERα transfection, biochanin A had no effect on mRNA abundance. In order to study the transcriptional control, a fragment of the 5′-flanking region of the *ApoA-1* gene was amplified and inserted in a firefly luciferase reporter plasmid. The reporter assay indicated that the transactivation of the *ApoA-1* promoter was induced by biochanin A in HepG2 cells transfected with the ERα expression plasmid. This induction was reduced by the anti-oestrogen ICI 182,780, whereas the inhibitors of protein kinase (PK) C, PKA, or mitogen-activated kinase (ERK) had no suppressive effect. The present study illustrated that biochanin A might up regulate hepatic apoA-1 mRNA expression through an ER-dependent pathway.

Biochanin A: Oestrogen receptors: Apolipoprotein A-1: Liver cancer cells

CVD comprises 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 to alleviate several key clinical risk factors for CVD[3].

HDL is synthesised in hepatic and intestinal cells and secreted as small particles containing phospholipids, non-esterified 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 (for a review, see Fielding & Fielding[4]).

Oestrogen receptor (ER) α is a member of nuclear hormone receptors for binding a wide range of hydrophobic molecules, such as steroid hormone and phyto-oestrogens. ERα is found in various tissues, including the liver, bone, heart and central nervous system[5]. 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 specific response elements for binding. The gene transcription machinery is then activated and the encoded mRNA is expressed.

Isoflavones are one group of the major phyto-oestrogens 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 (E_2)[6]. 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[7]. In addition, the plasma isoflavone concentration can be several thousand times greater than that of E_2[8]. They may compete for ER and display anti-oestrogenic effects, especially when endogenous oestrogen level is low. Biochanin A (5,7 dihydroxy-4′-methoxyisoflavone) can be isolated from red clover (*Trifolium pratense*), and is a nutraceutical for relieving postmenopausal symptoms. The oestrogenic activity of biochanin A is several orders of magnitude lower when compared with other structurally related isoflavones, such as genistein and daidzein[9].

Previous studies[10–12] have shown that HepG2 cells can be a viable model for apolipoprotein research except that these cells do not express ERα[13]. By using this cell model, the

Abbreviations: E_2, oestradiol; ER, oestrogen receptor; HNF, hepatocyte nuclear factor; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zolium bromide; PKA, protein kinase A; PKC, protein kinase C.

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present study was designed to investigate the regulatory mechanism of biochanin A on *ApoA-1*.

Materials and methods

Chemicals

Biochanin A was purchased from Sigma Chemicals (St Louis, MO, USA). PD98059, bisindolylmaleimide I and myristoylated PK inhibitor (PKI) 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 penicillin (50 U/ml) and streptomycin (50 μg/ml), and incubated at 37°C and 5 % carbon dioxide. At 3 d 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, UT, USA). Sub-confluent cell cultures were treated with isoflavone with dimethyl sulfoxide as the carrier solvent. The final concentration of the solvent was 0·1 % (v/v), and the control cultures received dimethyl sulfoxide only.

Luciferase reporter gene assay

Construction of ApoA-1-driven luciferase reporter plasmids. Fragments with 2361 bp (*ApoA-1*: −2068 to +293), 761 bp (468del: −468 to +293), 536 bp (243del: −243 to +293) and 333 bp (40del: −40 to +293) 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 were: pTA-*ApoA-1*-luc, 5′-CCG ACG CGT GGT ACC TTT CTA ACA GTT TTG-3′; pTA-468del-luc, 5′-TTC GAC GCG TAC TAA AGA AGA GCA CTG G-3′; pTA-243del-luc, 5′-TTT AAC GCG TCT GCA AGC CTG CAG CAC T-3′; pTA-40del-luc, 5′-AAG GAC GCG TGG CTG CAG ACA TAA ATA G-3′. 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 twenty-four-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 of 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 (Duke University, Durham, NC, USA). 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 the 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 reverse transcriptase-polymerase chain reaction 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 biochanin A. 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 μg total RNA using oligo-dT primers and Moloney murine leukaemia virus RT (M-MLV RT; USB Corporation, Cleveland, OH, USA). Target fragments were quantified by real-time PCR, and a DNA Engine Opticon™2 (MJ Research, Watertown, MA, USA) was employed for this assay. Taqman®–VIC® MGB probes and primers for apoA-1 and GAPDH (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 GAPDH (used as a reference housekeeping gene) were used to normalise the amount of total RNA amplified in each reaction. Relative gene expression data were analysed using the $2^{-\Delta\Delta C}$T method[14].

Western analysis

Cells were washed once by PBS (pH 7·4) and harvested into a 1·5 ml microtube with 0·5 ml lysis buffer (PBS, 1 % Nonidet® P-40 (NP-40), 0·5 % sodium deoxycholate and 0·1 % SDS). The lysis buffer contained protease inhibitors (PMSF (40 mg/l), aprotinin (0·5 mg/l), leupeptin (0·5 mg/l), 1·1 mM-EDTA and pepstatin (0·7 mg/l)). The harvested cells were then lysed with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) on ice for 30 s. The protein concentration of cell lysate was determined by Dc protein assay (BioRad, Richmond, CA, USA). Lysate protein (50 μg) was separated on 10 % SDS-PAGE and transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). For the Western analysis performed on culture medium, the volume loaded was normalised with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) absorbance reading. Anti-ApoA-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin primary (Sigma Chemicals) and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used for protein detection. An ECL Detection Kit (Amersham, Arlington Heights, IL, USA) provided the chemiluminescence substrate for horseradish peroxidase, and the targeted protein was visualised by autoradiography.

Measurement of cell viability

Cell number was assessed by MTT staining as described by Mosmann[15]. Briefly, HepG2 cells were seeded in a ninety-six-well plate and were transfected with ERα and

treated as described earlier. At the end of the treatment, 1500 μl MTT (1 mg/ml) were added to the cells and incubated at 37°C for 4 h. Cell viability was assessed with respect to the absorbance at 544 nm.

Statistical methods

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

Results

Effect of biochanin A on apolipoprotein A-1 mRNA expression

Cultures transfected with ERα had a dose-dependent increase in ApoA-1 mRNA abundance upon biochanin A treatment, whereas the apoA-1 expression in cells transfected with the empty vector was not affected by the same treatment (Fig. 1). Biochanin A at concentrations of 1 and 10 μM could induce 3- to 5-fold increases in mRNA abundance, whereas E_2 at 1 nM elicited an increase of about 7-fold. No induction was observed in empty vector-transfected cultures.

Response of ApoA-1 promoter to biochanin A in HepG2 cells expressing oestrogen receptor α

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 biochanin A at 0·5 μM or above (Fig. 2), and we observed increased activities ranging from 300 to 600 %. E_2 at the concentration of 1 nM also induced a 3-fold increase in the normalised luciferase activities compared with the control.

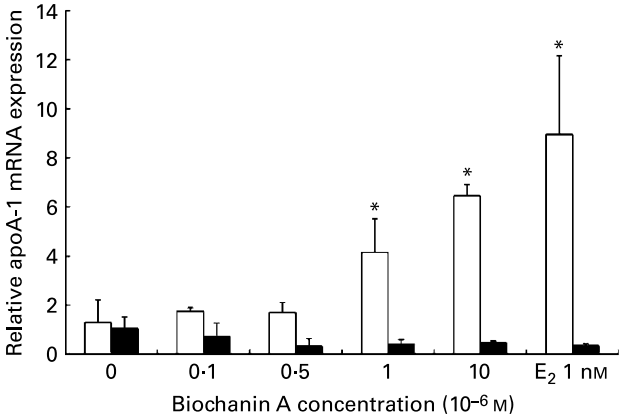

Fig. 1. Effect of biochanin A on ApoA-1 mRNA expression in the presence (■) and absence (□) of oestrogen receptor (ER) α in HepG2 cells. HepG2 cells were transfected with ERα expression plasmid or pcDNA3·1 vector. After 1 d, cells were treated with biochanin A or 1 nM-oestradiol (E_2). Total RNA was isolated and the ApoA-1 expression was measured. Values are means, with standard deviations represented by vertical bars. *Mean expression was significantly increased when compared with that of the control ($P<0{\cdot}05$).

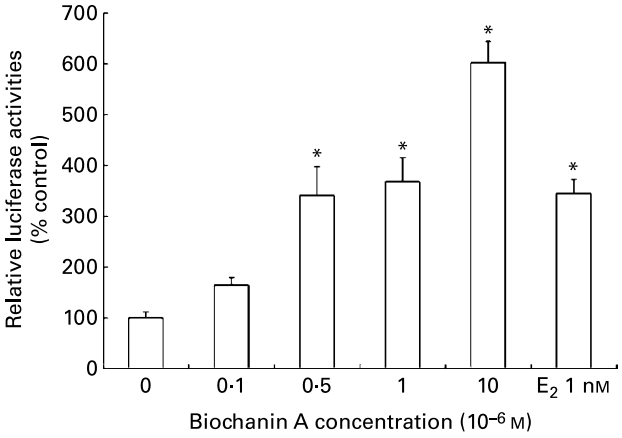

Fig. 2. Biochanin A increased *ApoA-1* promoter transactivation in HepG2 cells expressing oestrogen receptor (ER) α. HepG2 cells were plated and transfected with pTA-*ApoA-1*-luc, ERα expression plasmid and the control plasmid pRL. Cells were treated with biochanin A or oestradiol (E_2) for 24 h. Cell extracts were analysed for luciferase activity. Values are means, with standard deviations represented by vertical bars. *Mean expression was significantly increased when compared with that of the control ($P<0{\cdot}05$).

Effect of isoflavone on apolipoprotein A-1 protein

ApoA-1 expression at the protein level was also assessed in cell lysates and culture medium. However, the ApoA-1 protein in cell lysates or culture medium was not increased by biochanin A treatment (Fig. 3). It was possible that the translational machinery was unable to cope with the increased messages in this cell system.

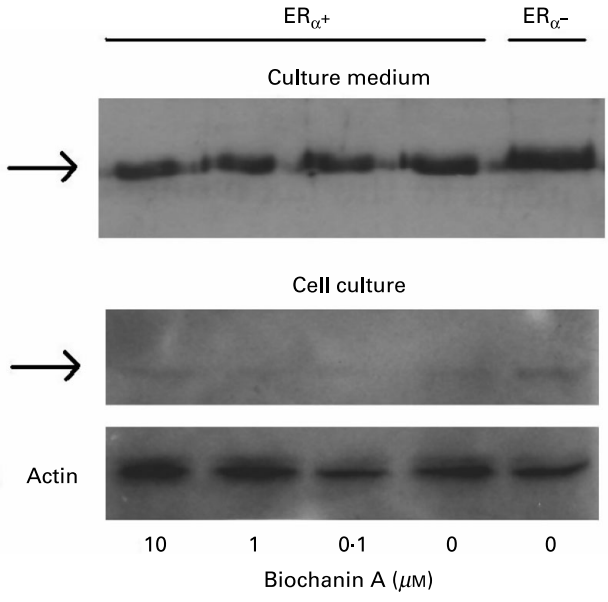

Fig. 3. Western analysis of ApoA-1 protein in cultures and culture medium under biochanin A treatment. HepG2 cells were transfected with oestrogen receptor (ER) α and treated with biochanin A. Western analysis was performed on cell lysates and culture medium. The autoradiographs are shown; the culture medium was normalised with the respective viable cell number (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zolium bromide (MTT) absorbance). The blots are representatives of two independent experiments with similar results.

Effect of protein kinase inhibitors and anti-oestrogen on ApoA-1 promoter-driven luciferase activities in cultures expressing oestrogen receptor α

Previous studies[16,17] have indicated the involvement of protein kinase A (PKA), protein kinase C (PKC) or mitogen-activated protein kinase in *ApoA-1* regulation. Protein kinase inhibitor (PKI) 14–22 amide, bisindolylmaleimide I and PD98059 are specific inhibitors for PKA, PKC and mitogen-activated protein kinase kinase, respectively. The inhibitor concentrations have been validated in HepG2 cells[16]. Compared with the control the administration of these inhibitors did not substantially decrease the *ApoA-1* promoter-driven gene transactivation (Fig. 4(A)). PD98059 could induce rather than suppress the promoter activity. When the pure anti-oestrogen ICI 182,730 was administered, luciferase activity was significantly ($P<0{\cdot}05$) reduced as demonstrated in Fig. 4(B). These data illustrated that ERα was involved in biochanin A-induced *ApoA-1* transcriptional activation.

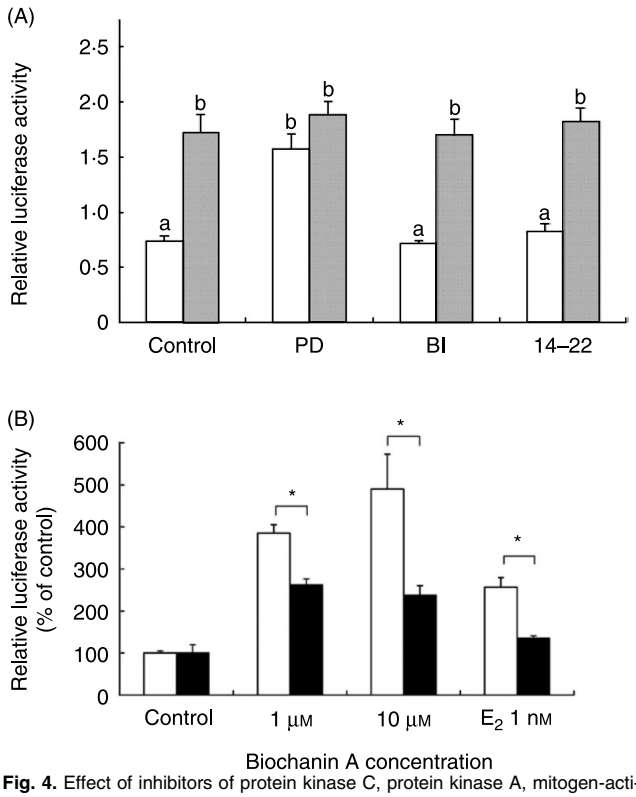

Fig. 4. Effect of inhibitors of protein kinase C, protein kinase A, mitogen-activated protein kinase and ICI 182,780 on biochanin A-induced *ApoA-1* promoter transactivation. HepG2 cells were transiently transfected with pTA-*ApoA-1*-luc plasmid and oestrogen receptor α expression plasmid for 24 h. (A) Following transfection, cells were pretreated for 1 h with 10 μM-PD 98 059 (PD), 10 μM-myristoylated protein kinase inhibitor (PKI) 14–22 amide (14–22) and 1 μM-bisindoylmaleimde I (BI). Biochanin A (1 μM; ▩) or dimethyl sulfoxide (□) was then administered. Values are means, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different ($P<0{\cdot}05$). (B) Biochanin A or 1 nM-oestradiol (E_2) was administered with (■) or without (□) pretreatment of ICI 182,780 for 1 h. Cell extracts were collected after 24 h for the determination of luciferase activity. Values are means, with standard deviations represented by vertical bars. *Mean activity was significantly decreased when compared with the biochanin A- or E_2-treated cultures ($P<0{\cdot}05$).

Determination of ApoA-1 promoter-driven luciferase activities in oestrogen receptor α-transfected HepG2 cells

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 biochanin A (Fig. 5(A)). The greatest response was observed in the pTA-*ApoA-1*-luc construct. However, the increases of the luciferase signals were similar among different biochanin A concentrations within the same construct in terms of the percentage induced. This indicated that no critical promoter segment was induced by biochanin A (Fig. 5(B)).

Investigation of ApoA-1 promoter-driven luciferase activities in HepG2 cells expressing oestrogen receptor β

Since ERβ might also activate oestrogen response element, we carried out another reporter gene assay to determine whether

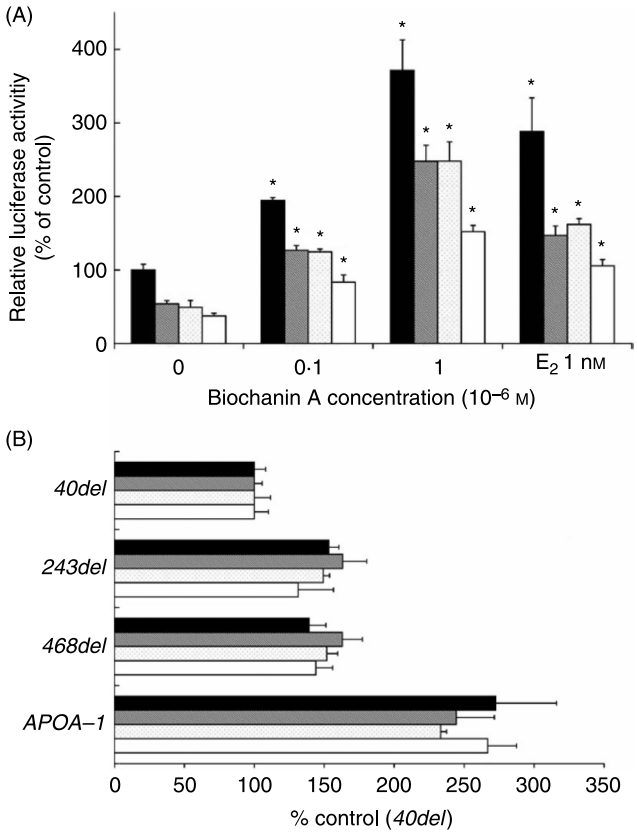

Fig. 5. Biochanin A-induced *ApoA-1* promoter activity profile. HepG2 cells were seeded in twenty-four-well culture plates and transfected with the serial truncation plasmid, oestrogen receptor α expression plasmid, and renilla luciferase plasmid. After 24 h of transfection, the cultures were treated with biochanin A for each construct. The cells were lysed and assayed for firefly and renilla luciferase activities. (A) One set of two experiments performed with comparable results. (■), *ApoA-1*; (▩), *468del;* (▩), *243del;* (□), *40del.* Values are means, with standard deviations represented by vertical bars. *Mean activity was significantly increased when compared with the control cultures ($P<0{\cdot}05$). (B) Replot of the values for each of the truncated *ApoA-1* promoter normalised with its own construct without biochanin A treatment. (■), 1 nM-Oestradiol; (▩), 1 μM-biochanin A; (▩), 0·1 μM-biochanin A; (□), 0 μM-biochanin A. Values are are means (n 3), with standard deviations represented by vertical bars.

the expression was also regulated by this ER isoform. Biochanin A at 10 μM showed a marginal elevation in the normalised luciferase activities (Fig. 6), and the increased activities were not deviated from the empty vector. The *ApoA-1* promoter-driven luciferase activity was not significantly increased by biochanin A in cells expressing ERβ.

Discussion

In the present study, we found that biochanin A up regulated apoA-1 mRNA expression in HepG2 cells expressing ERα, not ERβ. *ApoA-1* promoter-driven reporter gene assays supported that the up regulation was introduced by increased transcriptional activities. The induction pathway appeared to be independent of mitogen-activated kinase (ERK), PKA and PKC. Luciferase assays using the truncation reporter plasmids also did not reveal any critical elements lying between −40 and −2068 in the 5′-flanking region of the *ApoA-1* promoter.

17β-E_2 and genistein have been shown to increase the promoter activities of *ApoA-1* in HepG2 cells[18,19]. Similarly, the present study demonstrated that biochanin A activated both the ApoA-1 mRNA expression and *ApoA-1* promoter activity. The phyto-oestrogen biochanin A appeared to activate ERα for the induction of mRNA expression of ApoA-1, and the condition has not been established in the above-mentioned studies. It has been shown that the mitogen-activated kinase (ERK) activation pathway is increased in the up regulation of *ApoA-1* gene expression by genistein and E_2 in wild-type HepG2 cells[16]. Conversely, Beers *et al.*[17] have shown that overexpressing ERK1/2 suppresses rather than encourages the transcriptional activities. The present study indicated that inhibition of several signalling pathways including the mitogen-activated protein kinase, PKC and PKA pathways did not abolish the augmented *ApoA-1* transcription. Mitogen-activated protein kinase inhibitor by itself might even increase the transactivity, which could be contradictory to the above-mentioned observations. This suggested that the up regulation of *ApoA-1* transcriptional activity in the presence of ERα was probably not going through these signalling pathways.

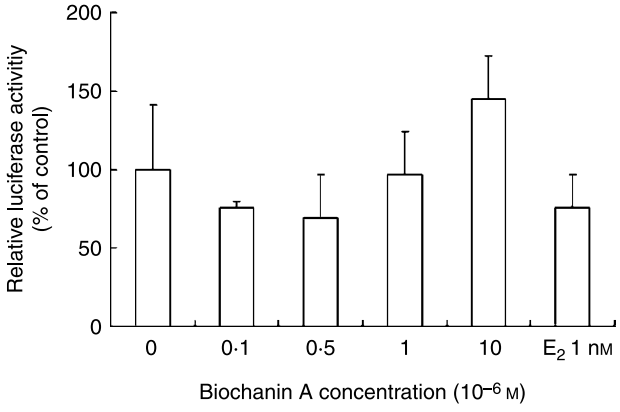

Fig. 6. Effect of biochanin A on *ApoA-1* promoter transactivation in HepG2 cells expressing oestrogen receptor (ER) β. HepG2 cells were plated and transfected with pTA-*ApoA-1*-luc, ERβ expression plasmid and the control plasmid pRL. Cells were treated with biochanin A or oestradiol (E_2) for 24 h. Cell extracts were analysed for luciferase activity. Values are means, with standard deviation represented by vertical bars.

The first 256 bp upstream in the 5′-flanking region is critical in *ApoA-1* regulation in HepG2 cells. Unlike the intestinal Caco-2 cells which require the segment −192 to −2052 for transcription, the segment between −41 and −256 is sufficient and specific for maximal *ApoA-1* transcription in HepG2 cells[20]. Previous studies have shown that the increase in *ApoA-1* gene expression by oestrogen and genistein is mediated through the −256 to −41 region of the *ApoA-1* promoter[18,19]. This region contains binding sites for three transcription factors, which are hepatocyte nuclear factors (HNF)-3β, HNF-4 and early growth response factor Egr-1. Sites at −214 to −192 and −169 to −146 have been shown to contain response elements for HNF-4[21] and HNF-3β[22], respectively. Binding sites for Egr-1 have also been located at −221 to −231 and −189 to −181[23]. Our findings did not support the notion that oestrogen increased ApoA-1 mRNA expression in HepG2 cells without ERα. With respect to our truncation reporter experiments performed in HepG2 cells expressing ERα, biochanin A treatment did not initiate a higher luciferase response than the control in cells transfected with reporter plasmids constructed by deleting sequences from −2068 to −40. Hence, the *cis*-acting DNA binding sites activated by biochanin A was apparently not present in this segment. Because an increase in promoter activity was observed in the reporter assays, biochanin A might activate an enhancer element or deactivate a repressor element in a region further upstream or downstream of the gene. A 48 bp enhancer element locating at the far distal region of *ApoA-1* (+8446/+8399) has been identified[24], which could be a possible activation pathway in the present study.

Oestrogen replacement therapy has long been used for controlling postmenopausal symptoms, including lowering blood cholesterol. Lamon-Fava *et al.*[18] have demonstrated that E_2 increases promoter activities of *ApoA-1* in HepG2 cells. An ER-independent pathway has also been described by Zhang *et al.*[25] for equine oestrogen in the up regulation of *ApoA-1* promoter activity. In contrast, oestrogen may repress ApoA-1 expression. Harnish *et al.*[13] observed that 100 nM-E_2 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.

Acknowledgements

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