Soya phytoestrogens, genistein and daidzein, decrease apolipoprotein B secretion from HepG2 cells through multiple mechanisms

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Diets containing the soya-derived phytoestrogens, genistein and daidzein, decrease plasma cholesterol in humans and experimental animals. The mechanisms responsible for the hypocholesterolaemic effects of these isoflavones are unknown. The present study was conducted to determine if genistein and daidzein regulate hepatocyte cholesterol metabolism and apolipoprotein (apo) B secretion in cultured human hepatoma (HepG2) cells. ApoB secretion was decreased dose-dependently by up to 63 % and 71 % by genistein and daidzein (100 μM; $P < 0.0001$) respectively. In contrast, no effect on apoAI secretion was observed. Cellular cholesterol synthesis was inhibited 41 % by genistein (100 μM; $P < 0.005$) and 18 % by daidzein (100 μM; $P < 0.05$), which was associated with significant increases in 3-hydroxy-3-methylglutaryl-CoA reductase mRNA. Cellular cholesterol esterification was decreased 56 % by genistein (100 μM; $P < 0.04$) and 29 % by daidzein (100 μM; $P < 0.04$); however, mRNA levels for acyl-CoA:cholesterol acyltransferase (ACAT) 1 and ACAT2 were unaffected. At 100 μM, both isoflavones equally inhibited the activities of both forms of ACAT in cells transfected with either ACAT1 or ACAT2. Genistein (100 μM) and daidzein (100 μM) significantly decreased the activity of microsomal triacylglycerol transfer protein (MTP) by 30 % and 24 % respectively, and significantly decreased MTP mRNA levels by 35 % and 55 %. Both isoflavones increased low-density lipoprotein (LDL)-receptor mRNA levels by 3- to 6-fold (100 μM; $P < 0.03$) and significantly increased the binding, uptake and degradation of ^{125}I-labelled LDL, suggesting that enhanced reuptake of newly secreted apoB-containing lipoproteins contributed to the net decrease in apoB secretion. These results indicate that genistein and daidzein inhibit hepatocyte apoB secretion through several mechanisms, including inhibition of cholesterol synthesis and esterification, inhibition of MTP activity and expression and increased expression of the LDL-receptor.

Key words: acyl-CoA: cholesterol acyltransferase, isoflavone, low-density lipoprotein-receptor, microsomal triacylglycerol transfer protein.

INTRODUCTION

Diets containing soya protein lower plasma cholesterol concentrations in humans and experimental animals [1–4]. Early studies in rabbits fed cholesterol-free diets demonstrated that isolated soya protein was one of the most hypocholesterolaemic proteins of various dietary plant and animal proteins [5,6]. Several mechanisms have been proposed to explain the hypocholesterolaemic effect of soya protein diets. In rabbits, soya protein enhanced plasma cholesterol turnover, increased faecal neutral and acidic steroid secretion, decreased cholesterol reabsorption and decreased the secretion of apolipoprotein (apo) B into plasma [7–9]. Soya protein has been linked to up-regulation of the low-density lipoprotein (LDL)-receptor in both animal [2] and human studies [10,11]. Nevertheless, the components of soya responsible for these effects have not been clearly identified.

Research to date has focused on both nutritive and non-nutritive components of soya protein and the possible mechanisms by which LDL cholesterol may be decreased. There are a number of biologically active compounds associated with soya protein, including soya fibre, phytic acid, saponins, trypsin inhibitors and isoflavones, which may contribute to the hypocholesterolaemic effects of soya protein [3]. Isoflavones, which are primarily found in soyabeans, are one class of phytoestrogen. The isoflavones most abundant in soya are genistein[5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one]anddaidzein[7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyrane-4-one]. Genistein and daidzein are derived from the hydrolysis of their glycoside forms present in soya products. Studies in cynomolgous monkeys examined mildly atherogenic diets containing isolated soya protein either with the isoflavones genistein and daidzein intact or containing only the trace amounts following isoflavone extraction. Soya protein enriched in isoflavones significantly decreased LDL and very-low-density lipoprotein (VLDL) cholesterol concentrations compared with the isoflavone-deficient diet [12–14]. The contribution of isoflavones to the cholesterol-lowering properties of soya protein has also been investigated in normal and hypercholesterolaemic men and women. Soya protein containing increased concentrations of isoflavones significantly decreased LDL cholesterol concentrations in a dose-responsive manner [15–18].

The cellular mechanisms whereby soya isoflavones exert their hypocholesterolaemic effects are not well understood. We hypothesized that genistein and daidzein inhibit hepatic assembly

Abbreviations used: apo, apolipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; CE, cholesteryl ester; FC, free cholesterol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; MEM, minimal essential medium; MTP, microsomal triacylglycerol transfer protein; PL, phospholipid; TC, total cholesterol; TG, triacylglycerol; VLDL, very-low-density lipoprotein.

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and secretion of apoB-containing lipoproteins and/or enhance the expression of hepatic LDL-receptors. Hepatic production of apoB-containing lipoproteins is regulated largely at post-transcriptional levels, as nascent apoB either associates with sufficient lipid to be secreted or is degraded intracellularly (reviewed in [19–21]). The assembly of apoB with lipid to form a secretion-competent particle is a complex process [19] and a number of factors play key roles, any of which may be modulated by isoflavones. Lipid availability is obligatory for apoB-containing lipoprotein assembly within the liver, a finding supported by *in vitro* studies demonstrating the necessity for the synthesis of triacylglycerol (TG) [22,23], phospholipid (PL) [24], cholesterol [25] and cholesteryl esters (CE) [26,27] for apoB secretion. Microsomal triacylglycerol transfer protein (MTP) is critical for lipoprotein assembly by catalysing the transfer of these lipids to the nascent apoB molecule as it translocates across the endoplasmic reticulum membrane [28,29].

Diets containing soya protein enhance the clearance of apoB-containing lipoproteins [8,30]. Receptor-mediated catabolism of apoB-containing lipoproteins occurs largely via LDL-receptors expressed on the hepatocyte plasma membrane. LDL-receptor number is regulated tightly by the cellular concentration of free cholesterol (FC) [31] and by hormonal factors, such as oestrogen [32,33]. However, the effects of the isoflavones on LDL-receptor regulation are not well understood.

The present study was designed to examine the mechanisms whereby genistein and daidzein, the most abundant phytoestrogens in soya, regulate hepatic lipid and apoB metabolism. Specifically, we investigated the effects of these isoflavones on the secretion of apoB and the expression and activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, acyl-CoA: cholesterol acyltransferase (ACAT), MTP and the LDL-receptor in the human hepatoma cell line HepG2. Our results demonstrate that both genistein and daidzein significantly decrease apoB secretion from HepG2 cells through inhibition of cholesterol synthesis, cholesterol esterification and MTP activity. In addition, enhanced expression and activity of the LDL-receptor contributed to a net decrease in hepatocyte apoB production.

EXPERIMENTAL

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown as described previously [34]. For experiments, HepG2 cells were plated in either 100-mm dishes or in 6-well (35 mm)-culture plates (Falcon Scientific, Mississauga, ON, Canada) and maintained in minimal essential medium (MEM) containing 5 % (v/v) human lipoprotein-deficient serum (LPDS). Genistein or daidzein (10–100 μM; Sigma, St. Louis, MO, U.S.A.), solubilized in DMSO (concentration did not exceed 0.5 %), were added to the dishes. At concentrations of genistein above 200 μM, cell viability began to decline, whereas concentrations of daidzein above 200 μM were insoluble in media.

AC29 cells, a Chinese-hamster ovary cell line lacking endogenous ACAT activity, stably transfected with African green monkey ACAT1 or ACAT2 cDNA [35], were kindly provided by Dr L. Rudel (Wake Forest University School of Medicine, Winston-Salem, NC, U.S.A.). Stable transfections were performed using pIRESneo vectors (ClonTech, Palo Alto, CA, U.S.A.) containing ACAT1 or ACAT2 cDNAs with a FuGENE 6 (Boehringer Mannheim, Roche Diagnostics, Laval, QC, Canada) to DNA ratio of 6 μl:1 μg, and selection was done in the presence of Geneticin [36]. Cells were maintained as described previously [35], and the isoflavones, solubilized in DMSO, were added to the media as described for HepG2 cells.

Metabolic labelling experiments

Cells were preincubated with genistein, daidzein or DMSO (control) for 24 h prior to metabolic labelling. For dose–response experiments, cells were pulsed for 10 min with 100 μCi/ml Tran^{35}S label (L-[^{35}S]methionine and L-[^{35}S]cysteine; 1000 Ci/mmol; ICN, Costa Mesa, CA, U.S.A.) and chased for 60 min [25]. For intracellular apoB degradation experiments, cells were pulsed for 10 min and chased for 0–120 min. Isoflavones or DMSO were present throughout the pulse and the chase. Media and cellular apoB were immunoprecipitated, resolved by SDS/PAGE and quantified as described previously [25]. Samples from dose–response experiments were also immunoprecipitated for apoAI using 2 μl/sample of anti-(human apoAI) antibody (Calbiochem, La Jolla, CA, U.S.A.) [36].

ApoB Western blotting

ApoB secretion into the media was measured by Western blotting. Briefly, HepG2 cells were incubated for 24 h with genistein and daidzein (50 and 100 μM), after which time the media were collected and cellular protein determined. In some experiments, cells were incubated for 24 h in the presence of the MTP inhibitor BMS 197636 (10 nM; provided by Bristol Myers Squibb, Princeton, NJ, U.S.A.) or with oleic acid (0.1 mM). Portions of media samples, on the basis of cell protein, were resolved by SDS/PAGE (4.5 % gels), and proteins were transferred electrophoretically on to nitrocellulose membranes. The membranes were blocked (16 h, 4 °C) with 5 % (w/v) non-fat dried milk in PBS containing 0.1 % Tween-20. The membranes were then incubated with a monoclonal antibody specific for human apoB (1D1; Lipoprotein and Atherosclerosis Group, Heart Institute Research Corporation, Ottawa, ON, Canada), followed by incubation with a peroxidase-conjugated anti-(mouse IgG) antibody (Amersham Biosciences, Oakville, ON, Canada). ApoB was detected using BM Chemiluminescence Blotting Substrate (Boehringer Mannheim). Quantification analysis of the developed films was performed using an Imaging Densitometer (GS-700; Bio-Rad Laboratories, Mississauga, ON, Canada). The absorbance was linear over the concentrations used.

Lipid mass, lipid synthesis and CE hydrolysis

Cellular TG, FC and total cholesterol (TC) were quantified using enzymic reagents from Boehringer Mannheim as described previously [27]. Cellular lipid results are reported as μg of cellular lipid (CE, TG or FC)/mg of cell protein. The incorporation of [1-^{14}C]oleic acid (Amersham Biosciences) or [1-^{14}C]acetic acid (Amersham Biosciences) into cellular lipids was determined as described previously [27]. Incorporation of [^{14}C]oleic acid into CE was used as a measure of whole-cell ACAT activity. CE hydrolysis was determined following a 24 h preincubation of cells with [^{14}C]oleic acid (in the absence of isoflavones) to label intracellular CE. This was followed by incubations of 0–24 h in the presence of the isoflavones and the ACAT inhibitor DuP 128 (20 μM; DuPont Merck Pharmaceutical, Wilmington, DE, U.S.A.). The rate of decrease in radiolabelled CE was determined as described previously [36].

ACAT1 and ACAT2 activities

The direct effect of genistein or daidzein on ACAT1 and ACAT2 activities was determined in AC29 cells expressing either enzyme.

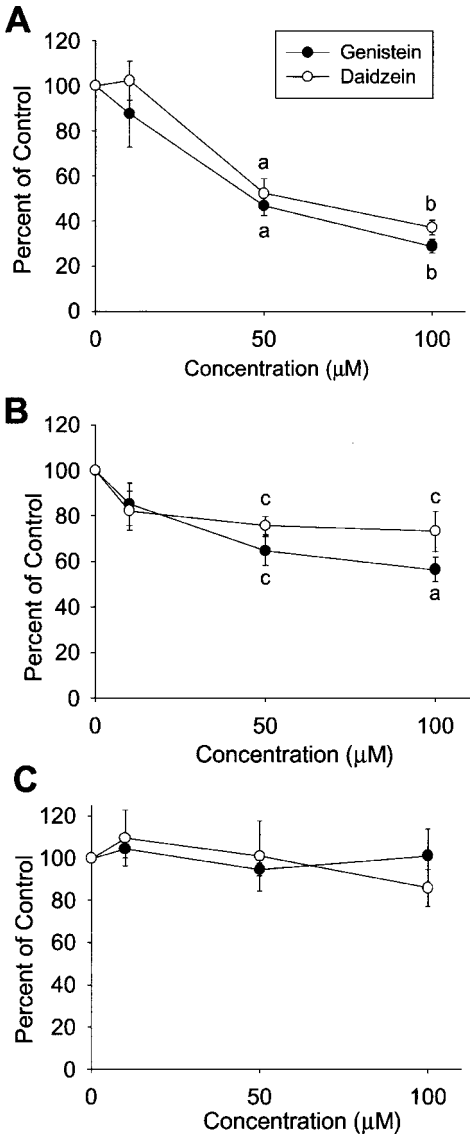

Figure 1 Effect of genistein and daidzein on apoB and apoAI secretion

HepG2 cells, preincubated in MEM containing 5% (v/v) LPDS and genistein or daidzein (at concentrations of 10–100 μM) for 24 h, were pulse-labelled with Tran^{35}S label for 10 min and chased for 60 min. The phytoestrogens were present throughout the pulse and chase. ApoB immunoprecipitated from the media (**A**) and cell lysates (**B**) is shown. (**C**) ApoAI immunoprecipitated from the media. Results are expressed as means $\pm$ S.E.M. ($n = 5$). $^{a}P < 0.003$, $^{b}P < 0.0001$ and $^{c}P < 0.05$ compared with control values.

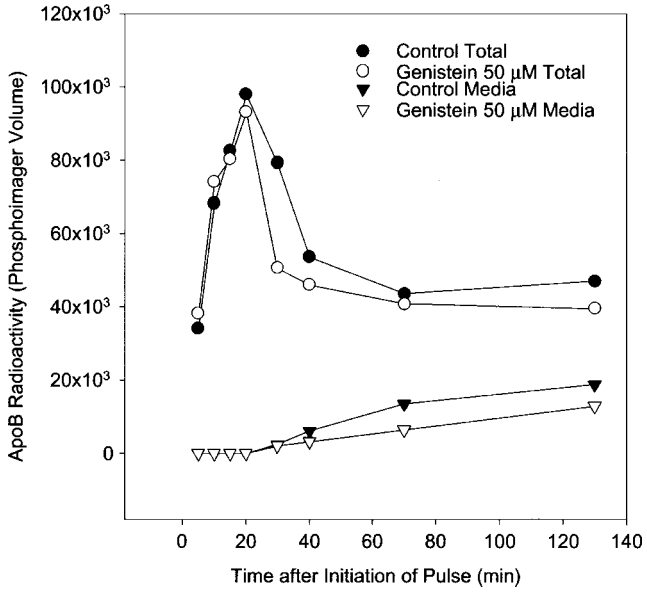

Figure 2 Effect of genistein on the secretion and intracellular degradation of apoB

HepG2 cells were incubated for 24 h in MEM containing 5% (v/v) LPDS in the absence (closed symbols) or presence (open symbols) of genistein (50 μM). Cells were then pulse-labelled for 10 min and chased for 0–120 min. Genistein was present throughout the pulse and the chase. ApoB100 radioactivity secreted into the media is represented by triangles. Total apoB100 radioactivity, determined as the sum of media and cellular apoB, is represented by circles.

In whole cells, the incorporation of [^{14}C]oleic acid into cellular CE was measured in the absence or presence of genistein or daidzein over 5 h, essentially as described above for HepG2 cells. In further experiments, microsomes were isolated from AC29 cells expressing either ACAT. The effect of genistein or daidzein on microsomal ACAT activity was determined in the presence of exogenous cholesterol [36]. Results were compared with those obtained using 10 μM of the ACAT inhibitors DuP 128 and CI-1011 (provided by Pfizer Inc., Ann Arbor, MI, U.S.A.).

MTP activity

MTP activity was assessed using an isotopic transfer assay as described previously [36]. Briefly, cell homogenates from HepG2 cells preincubated for 24 h in the absence or presence of genistein or daidzein were used as the MTP source. Results were compared with those obtained from cells incubated for 24 h with 10 nM of the MTP inhibitor BMS 197636. High-density lipoprotein (HDL)$_3$ [relative density (d) > 1.13] and LDL (1.019 < d < 1.063) were isolated from human plasma and the HDL$_3$ incubated with [1α,2α(n)-^{3}H]cholesterol (Amersham Biosciences) for 24 h at 37 °C. The MTP reaction mixture contained donor lipoproteins ([^{3}H]CE-containing HDL) and acceptor lipoproteins (LDL) in a ratio of 1:10 (on the basis of protein concentration), 50 μg of HepG2 cell homogenate and dialysis buffer [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 3 mM sodium azide] in a final volume of 50 μl. MTP-mediated [^{3}H]CE transfer was determined over 6 h at 37 °C. Radioactivity in the acceptor particles, reisolated by ultracentrifugation, was counted and the percentage transfer of CE was calculated [36].

Measurement of mRNA abundance

HepG2 cells were seeded in 100-mm plates in MEM supplemented with 10% (v/v) foetal bovine serum and grown to 70–80% confluency. Cells were incubated in the absence or presence of genistein or daidzein for 24 h in MEM supplemented with 5% (v/v) LPDS. Total RNA was isolated using Trizol reagent (Life Technologies, Mississauga, ON, Canada).

Custom oligonucleotide primers were generated corresponding to human apoB (GenBank® database accession number M10374) 5′-CCATTATGGACTTTCGAATATACCTGGGACAGTAC-CGTCCCTACCTCCCGGCGTCCA-3′, human LDL-receptor (GenBank® accession number L29401) 5′-CTCGCCACTTAGG-CAGTGGAACTCGAAGGCCGAGCAGGGGCTACTGTC-CCCGGTTCCAC-3′, human HMG-CoA reductase (GenBank®

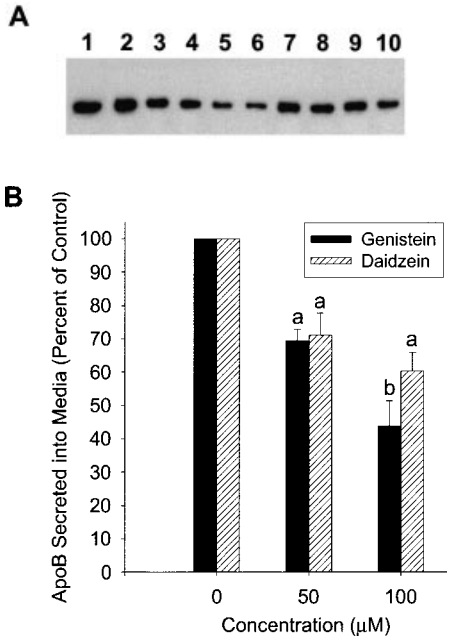

Figure 3 Effect of genistein and daidzein on apoB accumulation in the media of HepG2 cells

HepG2 cells were incubated for 24 h in MEM containing 5% (v/v) LPDS in the absence or presence of genistein or daidzein at concentrations of 50 and 100 μM. At the end of the incubation period, apoB secreted into the media was measured by Western blotting. (**A**) A representative Western blot. Lanes 1 and 2, media from control cells; lanes 3 and 4, cells incubated with 50 μM genistein; lanes 5 and 6, cells incubated with 100 μM genistein; lanes 7 and 8, cells incubated with 50 μM daidzein; and lanes 9 and 10, cells incubated with 100 μM daidzein. (**B**) Quantified data for genistein (solid bars) or daidzein (hatched bars) derived from each Western blot and expressed as means $\pm$ S.E.M. ($n = 5$). [a]$P < 0.0001$ and [b]$P < 0.002$ compared with control.

accession number NM_000859.1) 5′-CCAACAGGGATGGGCATATATCCAATAACATTCTCACAACAAGCTCCCATCCAAC-3′, human MTP (GenBank® accession number NM_000253) 5′-GGGTTTTAGACTCACGATACCACAAGCTAAACTCCCGGT-3′, human ACAT1 (GenBank® accession number L21934) 5′-ACAGTGAATTTCTGATTTCACTCAGGG-3′, human ACAT2 (GenBank® accession number AF099031) 5′-GTCCTGGTCCCCAGGTTTTGCAGGCAGAGAATCTGGTGGTGTCTGGGACTT-3′ and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank® accession number BC029618.1) 5′-CGGAAGGCCATGCCAGTGAGCTTCAATG-3′. Oligonucleotides (5 pmol) were 5′ end-labelled by incubating for 10 min at 37 °C with [γ-^{32}P]ATP (7000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase (Life Technologies; 10 units/μl), and were reisolated after filtering through quick spin centrifuge columns (Boehringer Mannheim). Oligonucleotide probes for the gene of interest together with the oligonucleotide probe for GAPDH were hybridized to total RNA (20 μg) from either control or treated cells and incubated for 10 min at 75 °C and then overnight at 55 °C [37]. Following hybridization, samples were incubated with 300 units of S1 nuclease (30 min, 37 °C; Boehringer Mannheim). After precipitation with ethanol, the probes were resolved by denaturing PAGE (19% gels). Bands were visualized using a phosphoimager and quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Data are expressed as band intensity for the gene of interest compared with that obtained for GAPDH.

Table 1 Effect of genistein and daidzein on cellular lipid mass

HepG2 cells were incubated in MEM containing 5% (v/v) LPDS with genistein or daidzein at concentrations of 50 and 100 μM for 24 h. Following incubation, lipids were extracted from cell monolayers and quantified by spectrophotometric assays. CE mass is calculated as the difference between TC and FC mass values. Values are means $\pm$ S.E.M. [a]$P < 0.05$, [b]$P < 0.02$ and [c]$P < 0.01$ compared with control.

	Cellular lipid mass (μg/mg of cell protein)		
Treatment	CE	FC	TG
Control	6.33 ± 0.71	15.73 ± 0.72	32.75 ± 2.25
Genistein (50 μM)	5.06 ± 0.47^{b}	13.74 ± 0.57^{c}	45.11 ± 2.44^{c}
Genistein (100 μM)	5.19 ± 0.43^{a}	13.60 ± 0.93^{b}	45.71 ± 2.69^{c}
Daidzein (50 μM)	5.15 ± 0.50^{a}	14.32 ± 0.58^{b}	35.38 ± 2.00
Daidzein (100 μM)	4.80 ± 0.70^{a}	13.63 ± 0.63^{c}	35.48 ± 2.25

^{125}I-labelled LDL binding and uptake studies

LDL ($1.019 < d < 1.063$) was isolated from human plasma and radiolabelled with ^{125}I using the iodine monochloride technique [38]. ^{125}I-labelled LDL binding, uptake and degradation studies were carried out as described previously [38].

Statistics

Experiments were performed at least three times and all conditions were determined in duplicate. Values are presented as means $\pm$ S.E.M. Statistical differences between means were determined by Student's t test. A P value < 0.05 was considered significant.

RESULTS

ApoB and apoAI secretion

HepG2 cells were preincubated with genistein and daidzein (10–100 μM) for 24 h prior to metabolic labelling experiments. As shown in Figure 1(A), genistein decreased the secretion of radiolabelled apoB into the media by 53% at 50 μM ($P < 0.003$) and 71% at 100 μM ($P < 0.0001$) compared with control. Daidzein decreased radiolabelled apoB secretion by 48% (50 μM; $P < 0.003$) and 63% (100 μM; $P < 0.0001$). At concentrations of 10 μM or less, neither isoflavone significantly affected apoB secretion. The decreased secretion in apoB observed with both isoflavones was accompanied by a dose-dependent decrease in cellular radiolabelled apoB, with up to 44% decrease at 100 μM genistein ($P < 0.003$) and 25% decrease at 100 μM daidzein ($P < 0.05$) (Figure 1B). ApoAI secretion from HepG2 cells was not significantly affected by genistein or daidzein at concentrations ranging from 10–100 μM (Figure 1C). Furthermore, the incorporation of Tran^{35}S label into total trichloroacetic acid ('TCA')-precipitable counts (cell plus media) was not significantly affected by genistein or daidzein treatment (results not shown). At the concentrations of isoflavones used in this study, there were no significant effects on total cell protein or viability (results not shown).

Intracellular apoB degradation in the presence of genistein (50 μM) was measured by pulsing cells for 10 min with Trans^{35}S label, followed by 0–120 min of chase. Media and cell lysates were collected throughout the pulse and the chase. Incorporation of label into apoB was unchanged during the pulse and until peak incorporation was reached 20 min after initiation of the pulse (Figure 2). However, in genistein-treated cells, apoB degradation was increased by 36% at 30 min and 16% at the end of the chase (Figure 2, Total). Consequently, secretion of

Table 2 Effect of genistein and daidzein on [^{14}C]oleic acid and [^{14}C]acetic acid incorporation into cellular lipids

HepG2 cells treated with 50 and 100 μM genistein or daidzein were incubated with 0.08 μCi [^{14}C]oleic acid or 0.5 μCi [^{14}C]acetic acid for 5 h. The 5 h incubation was initiated immediately after addition of the phytoestrogens (0–5 h) or following a 19 h incubation (19–24 h) with the phytoestrogens. At the end of the incubation, the cellular lipids were extracted and analysed by TLC as described in the Experimental section. CE, TG and PL are expressed as nmol [^{14}C]oleic acid incorporated/mg of cell protein, and TC is expressed as pmol [^{14}C]acetic acid incorporated/mg of cell protein. Values are means ± S.E.M. [a]$P < 0.05$, [b]$P < 0.02$ and [c]$P < 0.01$ compared with control.

	Cellular lipids			
Treatment	CE	TG	PL	TC
Phytoestrogen preincubation (0 h)				
Control	2.31 ± 0.22	83.30 ± 6.81	35.94 ± 1.88	253.7 ± 12.8
Genistein (50 μM)	2.14 ± 0.14	99.79 ± 10.36[a]	33.06 ± 1.10	234.3 ± 15.7
Genistein (100 μM)	2.29 ± 0.18	100.67 ± 4.86[b]	31.33 ± 0.22	214.4 ± 15.5
Daidzein (50 μM)	2.09 ± 0.41	99.23 ± 7.33[b]	34.84 ± 0.68	306.9 ± 19.5
Daidzein (100 μM)	2.18 ± 0.19	108.63 ± 1.99[b]	32.83 ± 1.93	299.5 ± 8.10
Phytoestrogen preincubation (19 h)				
Control	3.07 ± 0.15	80.48 ± 5.44	32.77 ± 1.56	752.7 ± 48.8
Genistein (50 μM)	1.38 ± 0.12[a]	99.62 ± 5.68[b]	31.12 ± 1.62	483.1 ± 6.20[a]
Genistein (100 μM)	1.34 ± 0.15[a]	107.28 ± 3.65[a]	29.37 ± 0.56	445.9 ± 16.2[b]
Daidzein (50 μM)	2.20 ± 0.32[c]	86.72 ± 5.49[c]	32.06 ± 1.06	664.7 ± 37.4[a]
Daidzein (100 μM)	2.19 ± 0.12[c]	89.98 ± 8.28[a]	31.64 ± 1.27	614.7 ± 19.8[a]

Table 3 Effect of genistein and daidzein on the activities of ACAT1 and ACAT2

ACAT1 and ACAT2 activities were determined using AC29 cells stably transfected with African green monkey ACAT1 or ACAT2 cDNA. For microsomal assays, genistein and daidzein (50 and 100 μM) were added in the presence of exogenous cholesterol. Values are means ± S.E.M. [a]$P < 0.006$, [b]$P < 0.00008$ and [c]$P < 0.02$ compared with control.

	Cellular ACAT activity (% of control)		Microsomal ACAT activity (% of control)	
Treatment	ACAT1	ACAT2	ACAT1	ACAT2
Control	100	100	100	100
Genistein (50 μM)	94.0 ± 5.5	91.9 ± 1.8[a]	75.0 ± 5.0[b]	74.8 ± 8.7[c]
Genistein (100 μM)	82.0 ± 4.3[a]	78.2 ± 2.9[b]	61.6 ± 5.6[a]	62.3 ± 6.8[a]
Daidzein (50 μM)	88.8 ± 2.2[a]	90.6 ± 2.4[a]	91.9 ± 4.3[b]	88.0 ± 9.3
Daidzein (100 μM)	89.5 ± 3.1[c]	85.3 ± 2.1[b]	79.7 ± 5.7[c]	77.7 ± 8.1[c]
Dup 128 (10 μM)			0	0
CI-1011 (10 μM)			2.3	0.4

newly synthesized apoB into the media was decreased by 31 % in genistein-treated cells (Figure 2, Media). Similar results were observed for daidzein (results not shown).

The effect of genistein and daidzein on the accumulation of apoB mass in the media during a 24 h incubation was determined by Western blotting (Figure 3). Genistein decreased media apoB by 30 % (50 μM; $P < 0.0001$) and 56 % (100 μM; $P < 0.002$), whereas daidzein decreased media apoB by 29 % (50 μM; $P < 0.0001$) and 39 % (100 μM; $P < 0.0001$), confirming the results obtained by pulse–chase experiments. In cells incubated with 0.1 mM oleic acid, the increased secretion of apoB into the media was decreased by genistein and daidzein to a similar extent as in cells incubated in the absence of oleic acid (results not shown).

Cellular lipid mass

The effect of genistein and daidzein on the mass of intracellular lipids in HepG2 cells was determined following 24 h incubations with either isoflavone. As shown in Table 1, genistein and daidzein significantly decreased the mass of CE by 18–24 % ($P < 0.05$). FC mass was also decreased and ranged from 9–14 % ($P < 0.01$). TG mass was unaffected by daidzein; however, genistein significantly increased TG by approx. 40 % ($P < 0.01$) compared with control. Little or no dose–response relationship was observed for changes in lipid mass.

Cellular lipid biosynthesis, ACAT activities and CE hydrolysis

Incorporation of [^{14}C]oleic acid or [^{14}C]acetic acid into cellular lipids was carried out over 5 h, either immediately or following a 19 h preincubation with genistein or daidzein. As shown in Table 2, with no preincubation, genistein and daidzein had no significant effect on the incorporation of [^{14}C]oleic acid into CE or PL. The incorporation of oleic acid into TG was significantly increased by both genistein and daidzein (21–30 %; $P < 0.02$). After a 19 h preincubation, genistein decreased the incorporation of [^{14}C]oleic acid into CE by 55 % (50 μM; $P < 0.05$) and 56 % (100 μM; $P < 0.05$), whereas daidzein decreased cholesterol esterification by 28 % at both doses ($P < 0.01$). Incorporation of [^{14}C]oleic acid into TG was increased by 24–33 % ($P < 0.05$) and 8–12 % ($P < 0.05$) by genistein and daidzein respectively. Neither isoflavone affected [^{14}C]oleic acid incorporation into PL. The incorporation of [^{14}C]acetic acid into TC was unaffected by a 5 h preincubation with either isoflavone (Table 2). However, following a 19 h preincubation, genistein decreased TC synthesis by 36 % (50 μM; $P < 0.05$) and 41 % (100 μM; $P < 0.02$) and daidzein decreased TC synthesis by 12 % (50 μM; $P < 0.05$) and 18 % (100 μM; $P < 0.05$).

In light of the discovery and characterization of a second ACAT enzyme, ACAT2, and the idea that ACAT2 may preferentially provide CEs for lipoprotein assembly (reviewed in [39]), we determined whether the isoflavones may selectively

Table 4 Effect of genistein and daidzein on MTP activity and apoB secretion

MTP activity was determined by [^{3}H]CE transfer from donor to acceptor lipoproteins, as described in the Experimental section. HepG2 cell homogenates were used as the MTP source. MTP activity was assessed in the absence or presence of genistein or daidzein (50 and 100 μM). For comparison, HepG2 cells were also incubated with the MTP inhibitor BMS 197636. ApoB secretion into the media over 24 h was determined as described in the Experimental section. Values are means $\pm$ S.E.M. $^aP < 0.0001$, $^bP < 0.002$ and $^cP < 0.04$ compared with control.

Treatment	MTP activity (% of control)	ApoB secretion (% of control)
Control	100 ± 11.4	100 ± 5.9
Genistein (50 μM)	76.1 ± 4.7^a	69.5 ± 3.4^b
Genistein (100 μM)	71.5 ± 6.4^b	43.8 ± 7.6^b
Daidzein (50 μM)	86.5 ± 6.4^c	71.0 ± 6.7^c
Daidzein (100 μM)	76.4 ± 9.1^c	60.5 ± 5.5^b
BMS 197636 (10 nM)	60.2 ± 6.7^c	46.8 ± 7.8^b

inhibit ACAT2 activity. AC29 cells stably transfected with either ACAT1 or ACAT2 cDNA were treated with either genistein or daidzein (50 and 100 μM). The isoflavones decreased cholesterol esterification in cells expressing either ACAT1 or ACAT2 to the same extent, as determined by assays using intact cells or isolated microsomes (Table 3).

CE hydrolysis was determined in HepG2 cells incubated with genistein or daidzein at concentrations of 50 and 100 μM. The rate of CE hydrolysis was decreased 17 ± 4.3% by 100 μM genistein ($P < 0.05$), but was unaffected by 50 μM genistein or either dose of daidzein (results not shown).

MTP activity

MTP activity in cell homogenates was determined as the transfer of [^{3}H]CE from donor to acceptor lipoproteins. As shown in Table 4, genistein decreased the transfer of [^{3}H]CE by 24% (50 μM; $P < 0.0001$) and 29% (100 μM; $P < 0.002$). Daidzein decreased the transfer of [^{3}H]CE to a lesser extent [14% at 50 μM ($P < 0.04$) and 24% at 100 μM ($P < 0.04$)]. The corresponding decreases in apoB secretion induced by 50 and 100 μM genistein or daidzein were 31%, 56%, 29% and 40% respectively. By comparison, the MTP inhibitor BMS 197636 inhibited the transfer of [^{3}H]CE by 40% ($P < 0.04$), which was associated with a 53% decrease in apoB secretion.

HepG2 cell mRNA content

The abundances of specific mRNAs were determined in HepG2 cells following 24 h incubation with either isoflavone. As shown in Table 5, LDL-receptor mRNA levels were significantly increased 3.1- and 5.7-fold by genistein (50 and 100 μM respectively) and 2.3- and 3.5-fold by daidzein (50 and 100 μM respectively). HMG-CoA reductase mRNA was significantly increased 1.2- and 1.5-fold by the 100 μM dose of each isoflavone. In contrast, MTP mRNA levels were significantly decreased by genistein (38% and 55% at 50 and 100 μM respectively; $P < 0.0001$). Daidzein also decreased MTP mRNA by 21% at 50 μM ($P < 0.03$) and by 35% at 100 μM ($P < 0.001$). ApoB, ACAT1 and ACAT2 mRNA levels were not significantly changed by either isoflavone compared with control. GAPDH mRNA levels were unchanged (results not shown).

Binding, uptake and degradation of ^{125}I-labelled LDL

HepG2 cells were preincubated with genistein and daidzein (50 or 100 μM) for 24 h prior to determining the cellular binding, uptake and degradation of ^{125}I-labelled LDL at 37 °C. As shown in Figure 4, genistein significantly increased cellular ^{125}I-labelled LDL binding by 1.7- and 2.1-fold (50 and 100 μM respectively; $P < 0.007$), uptake by 1.3-fold (50 μM; $P < 0.05$) and 1.6-fold (100 μM; $P < 0.00007$), and degradation by 1.5-fold (50 μM; $P < 0.0004$) and 1.6-fold (100 μM; $P < 0.05$). Daidzein also significantly increased cellular binding by 1.6- and 2.3-fold (50 and 100 μM respectively; $P < 0.00007$), uptake by 1.3-fold (50 μM; $P < 0.0004$) and 1.6-fold (100 μM; $P < 0.00007$), and degradation by 1.4-fold (50 μM; $P < 0.05$) and 1.7-fold (100 μM; $P < 0.0004$).

DISCUSSION

Soya-derived phytoestrogens, which consist primarily of the isoflavones genistein and daidzein, exert anti-atherogenic effects at multiple levels [40,41]. These include improvements in arterial compliance, decreased oxidation of LDL, inhibition of smooth-muscle cell proliferation and decreased platelet activation and aggregation. Beneficial effects of soya isoflavones on plasma lipid and lipoprotein levels have been demonstrated in both animal and human studies [12–18]. In the present study, we have elucidated some of the mechanisms responsible for the hypocholesterolaemic activity of the soya isoflavones.

Our results support the hypothesis that the effects of the soya isoflavones on apoB metabolism are due to decreased hepatic apoB production. We have shown for the first time that apoB secretion was significantly decreased by both genistein and daidzein in a dose-dependent manner, whereas apoAI secretion was unchanged. Our pulse–chase experiments clearly demonstrated that apoB synthesis was unaffected by the isoflavones and, therefore, are consistent with the established concept that, under most conditions, regulation of apoB secretion from HepG2 cells occurs post-translationally [19–21]. ApoB not incorporated into a secretion-competent lipoprotein is targeted for intracellular proteolytic degradation. The decreases in newly synthesized, cellular and secreted apoB demonstrated in the present study indicated enhanced apoB degradation in isoflavone-treated cells.

A novel observation in this study was the significant decrease in both the expression and activity of MTP by genistein and daidzein. MTP has been shown to be essential for the secretion of apoB-containing lipoproteins [29] by mediating the transfer of TG, CE and PL to nascent apoB molecules [28]. At the higher doses of genistein and daidzein, MTP activity was inhibited by 29% and 24% respectively. This level of inhibition probably makes a significant contribution to the observed decrease in apoB secretion. In control experiments, we determined that concentrations of the MTP inhibitor BMS 197636, which inhibited MTP activity to a similar extent as the isoflavones, were associated with significant decreases in apoB secretion. Furthermore, genistein and daidzein decreased MTP mRNA abundance by up to 55%. However, given that the relatively long half-life of the MTP protein is 4.4 days [42], the contribution of decreased expression to the decreased MTP activity over 24 h would be modest. Nevertheless, this result implies that with long-term genistein or daidzein treatment a decreased MTP expression would result in a significant impact on MTP activity and thus apoB secretion. The molecular mechanism linking isoflavones to the transcriptional regulation of MTP remains to be determined.

MTP inhibition has been shown to cause increases in hepatic triacylglycerol concentrations [29]. Whether the increased TG mass we observed with genistein and the increased TG synthesis

Table 5 Effect of genistein and daidzein on mRNA levels

Cultured HepG2 cells were incubated with genistein or daidzein at 50 and 100 μM for 24 h. Total RNA was extracted from the cells and quantified as described in the Experimental section. The relative mRNA levels for each phytoestrogen were normalized to each control mRNA level and expressed as the means ± S.E.M. Number (n) of experiments are shown in parentheses. [a]$P < 0.03$, [b]$P < 0.001$ and [c]$P < 0.0001$ compared with control. HMG-CoAR, HMG-CoA reductase.

	mRNA levels (relative to control)					
Treatment	ApoB ($n = 3$)	LDL-receptor ($n = 4$)	HMG-CoAR ($n = 4$)	MTP ($n = 3$)	ACAT1 ($n = 3$)	ACAT2 ($n = 5$)
Control	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.08
Genistein (50 μM)	0.98 ± 0.04	3.12 ± 0.52[b]	0.94 ± 0.06	0.62 ± 0.03[c]	0.91 ± 0.09	1.01 ± 0.16
Genistein (100 μM)	0.83 ± 0.04	5.73 ± 0.42[c]	1.23 ± 0.10[a]	0.45 ± 0.05[c]	1.08 ± 0.13	0.94 ± 0.16
Daidzein (50 μM)	0.92 ± 0.03	2.32 ± 0.16[c]	1.02 ± 0.07	0.79 ± 0.08[a]	0.97 ± 0.09	1.10 ± 0.16
Daidzein (100 μM)	0.83 ± 0.13	3.46 ± 0.27[a]	1.45 ± 0.13[b]	0.65 ± 0.09[b]	0.98 ± 0.05	1.07 ± 0.21

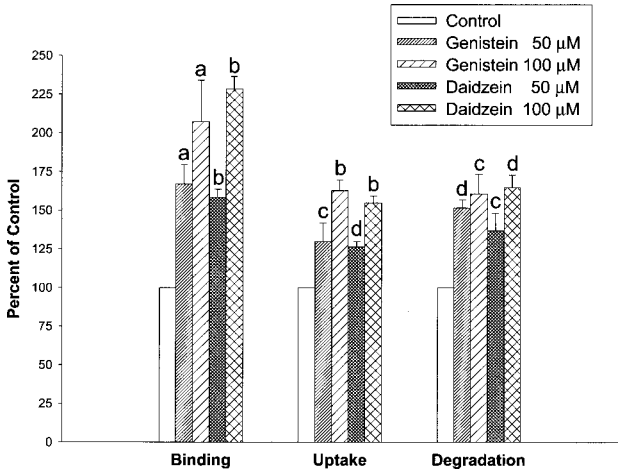

Figure 4 Effect of genistein and daidzein on LDL-receptor activity

HepG2 cells were incubated for 24 h with DMSO (control) genistein (50 and 100 μM) or daidzein (50 and 100 μM). ^{125}I-labelled LDL cellular binding, uptake and degradation were determined following the 24 h incubation. Genistein and daidzein were present throughout the ^{125}I-labelled LDL binding, uptake and degradation studies. Values are percentages of control and are expressed as means ± S.E.M. ($n = 4$). The absolute values for control binding, uptake and degradation are 13.6 ± 2.3, 123.7 ± 4.3 and 99.7 ± 22.7 ng of ^{125}I-labelled LDL/mg of cell protein respectively. [a]$P < 0.007$, [b]$P < 0.00007$, [c]$P < 0.05$ and [d]$P < 0.0004$ compared with control.

observed with both isoflavones is related to MTP inhibition is unknown. Haghpassand et al. [43] have shown that inhibition of MTP activity did not affect triacylglycerol synthesis in HepG2 cells. Similarly, we found that treatment of HepG2 cells with the MTP inhibitor BMS 197636, at concentrations resulting in the same extent of MTP inhibition as that induced by the isoflavones, had no effect on TG synthesis (results not shown). The increased TG synthesis may be related to ACAT inhibition, as we have reported previously [27] that the ACAT inhibitor DuP 128 increased HepG2 cell TG synthesis in a similar manner.

Soya protein-derived isoflavones may decrease plasma cholesterol levels by enhancing hepatic expression of LDL-receptors thereby increasing VLDL and/or LDL clearance. Increased plasma clearance of apoB-containing lipoproteins has been demonstrated in humans and rabbits fed soya protein-containing diets [8,9,30]. Furthermore, soya-containing diets with high isoflavone concentrations were shown to increase mononuclear cell LDL-receptor mRNA levels in hypercholesterolaemic human subjects [10,11]. Our results demonstrating that both isoflavones increase LDL-receptor expression and activity are entirely consistent with the concept that the net secretion of apoB from HepG2 cells is decreased due to cellular reuptake of newly secreted lipoproteins. The importance of the LDL-receptor in hepatocyte apoB secretion was highlighted by Twisk et al. [44], who observed that apoB secretion from hepatocytes derived from LDL-receptor knockout mice was 3.5-fold higher than that from wild-type hepatocytes. Kinetic modelling analyses indicated that LDL-receptor-mediated reuptake of newly secreted VLDL and presecretory degradation of newly synthesized apoB-containing lipoproteins resulted in a net decrease in apoB secreted from wild-type hepatocytes. The role of the LDL-receptor in mediating isoflavone-induced decrease in plasma cholesterol in mice was reported by Kirk et al. [45]. Diets enriched in genistein and daidzein decreased plasma cholesterol and atherosclerosis in wild-type mice but not in LDL-receptor-deficient mice.

Genistein and daidzein inhibited cellular cholesterol synthesis and cholesterol esterification. However, a preincubation of 19 h was required to observe inhibition of both activities. Although it is possible that the decrease in cholesterol synthesis was secondary to ACAT inhibition, we also observed a small, but significant, decrease in cellular FC mass. Furthermore, HMG-CoA reductase mRNA levels were significantly increased, similar to the compensatory increase observed following incubation of HepG2 cells with HMG-CoA reductase inhibitors [25]. Collectively, these results suggest that a metabolite(s) of genistein or daidzein functions as inhibitors of cholesterol synthesis and cholesterol esterification. We have shown previously *in vitro* and *in vivo* [25,27,46] that inhibition of HMG-CoA reductase or ACAT decreases the assembly and secretion of hepatic apoB-containing lipoproteins.

The extent to which decreased cholesterol synthesis or esterification by genistein and daidzein contribute to the decreased apoB secretion is difficult to determine. Cholesterol synthesis inhibition in HepG2 cells by genistein and daidzein (12–40 %) is less than that produced by the HMG-CoA reductase inhibitor atorvastatin (96 %), even though the decrease in apoB secretion by the isoflavones was greater (60–70 %) than that induced by atorvastatin (36 %) [25]. Similarly, ACAT inhibition in HepG2 cells by the isoflavones (30–50 %) is less than that produced by the ACAT inhibitor CI-1011 in HepG2 cells (approx. 80 %), even though the decrease in apoB secretion by the isoflavones was greater (60–70 %) compared with CI-1011 (45 %) [27]. Inhibition of hepatic ACAT has also been demonstrated for other flavonoids structurally similar to genistein and daidzein, namely naringenin and hesperetin [36] and baicalein [47]. Whether inhibition of ACAT and apoB

secretion are general features of flavonoids remains to be determined.

Genistein and daidzein equally inhibited CE synthesis in AC29 cells transfected with either ACAT1 or ACAT2. We speculated that the isoflavones may preferentially inhibit the activity of ACAT2, since it has been proposed that this form of the enzyme specifically provides CE for lipoprotein synthesis within the endoplasmic reticulum of the liver and intestine [39]. However, we observed no such specificity. Furthermore, the isoflavones had no effect on the mRNA abundances of ACAT1 or ACAT2. This contrasts with our recent findings [36] that the citrus flavonoids naringenin and hesperetin selectively decreased the expression of ACAT2.

Genistein is a known tyrosine kinase inhibitor with an IC_{50} of 20 μM, whereas daidzein does not inhibit tyrosine kinases at the concentrations used in the present study [48]. Despite this, daidzein, like genistein, inhibited apoB secretion, cholesterol synthesis, ACAT activity, MTP activity and expression as well as enhancing LDL-receptor expression. Therefore it is unlikely that the ability of genistein to inhibit tyrosine kinases is solely responsible for the ability of this isoflavone to inhibit apoB secretion.

The isoflavone-induced increases in LDL-receptor mRNA may be due to the oestrogenic activity of these compounds. Genistein and daidzein are weak oestrogenic compounds with 8 % and 3 % of oestradiol activity respectively [49]. Oestrogen has been shown to increase hepatic LDL-receptor expression in animal models [32] and increase LDL-receptor transcription in HepG2 cells [33]. On the other hand, it is unlikely that the oestrogenic action of these isoflavones is directly responsible for their effects on apoB secretion and cholesterol esterification in HepG2 cells observed in the present study. Oestrogen has been reported to cause no change [50] or even increase [51] apoB secretion in HepG2 cells. Furthermore, oestrogen treatment results in no change [52] or an increase [53] in hepatic ACAT activity.

Our results are consistent with studies conducted in animals and humans [12–18,41], showing decreased plasma cholesterol levels following administration of diets containing soya protein rich in isoflavones. However, the contribution of the isoflavones themselves to the modulation of plasma lipids has not been clearly established. Several human studies demonstrated no lipid-lowering effects of isolated isoflavones [54–56]. Furthermore, diets supplemented with an isoflavone extract from red clover, enriched in genistein and daidzein, did not result in any changes in plasma lipids (reviewed in [41]). It is possible that the bioavailability of the isoflavones is limited when provided in extract form. However, Setchell et al. [57] recently demonstrated that the maximum concentrations achieved in plasma, following single 50 mg oral doses of either genistein or daidzein to healthy young women, were 1.26 ± 0.27 and 0.76 ± 0.12 μM (means $\pm$ S.E.M.) respectively. Since isoflavones are subject to extensive first-pass clearance by the liver, it is feasible that human hepatocytes are exposed to isoflavone concentrations similar to those used in the present study. However, the possibility remains that another component of soya is required for the isoflavones to exert their effects on plasma lipid concentrations. Differences in the systemic bioavailabilities of genistein and daidzein [57] may also be reflected in our cell culture studies. We cannot rule out the possibility that any differences in the extent of the effects we observe are due to differences in the uptake and metabolism of genistein and daidzein in HepG2 cells.

In conclusion, the soya phytoestrogens genistein and daidzein significantly decreased the hepatic secretion of apoB-containing lipoproteins, an effect mediated by decreases in cholesterol synthesis, cholesterol esterification and MTP activity and enhanced expression of LDL-receptors. Many forms of hypercholesterolaemia are characterized by hepatic overproduction of apoB-containing lipoproteins [58]. Our results demonstrate that compounds such as genistein and daidzein may prove to be useful for treatment of these lipoprotein disorders. Furthermore, our findings suggest a new therapeutic paradigm, whereby simultaneous modulation of several key regulatory steps in the assembly and secretion of apoB-containing lipoproteins can be accomplished by one compound.

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