Dietary proteins with high isoflavone content or low methionine–glycine and lysine–arginine ratios are hypocholesterolaemic and lower the plasma homocysteine level in male Zucker fa/fa rats

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It has previously been demonstrated that soya protein, which contains isoflavones and low methionine–glycine and lysine–arginine ratios, has a hypocholesterolaemic effect. In the present study, the hypocholesterolaemic effects of an isoflavone-enriched casein diet (HDI) and a single-cell protein-based diet (SCP) devoid of isoflavones but with low methionine–glycine and lysine–arginine ratios were investigated in obese Zucker rats after 6 weeks of feeding. The control diet contained casein, which has high ratios of methionine–glycine and lysine–arginine. HDI and SCP feeding reduced the concentrations of total cholesterol and cholesteryl esters in plasma and liver, and changed the fatty acid composition of the hepatic cholesteryl esters. Faecal cholesterol and bile acid levels were markedly higher in SCP-fed rats than in controls, whereas HDI feeding had only minor effects. However, both HDI and SCP feeding increased the hepatic gene expression of cholesterol 7α hydroxylase. In contrast, the hepatic acyl-CoA synthetase and acyl-CoA:cholesterol acyltransferase activities and the gene expression of the LDL receptor were increased by HDI, but not by SCP feeding. The present results suggested that the cholesterol-lowering effect of SCP was related to the enterohepatic circulation, whereas HDI seemed to lower the plasma cholesterol via the circulation. Plasma homocysteine level was reduced in rats fed HDI and SCP compared to rats fed casein. In summary, diets enriched in isoflavones or containing proteins with low methionine–glycine and lysine–arginine ratios lowered the plasma cholesterol and homocysteine levels, changing the plasma profile from atherogenic to cardioprotective.

Amino acids: Bile acids: Cholesteryl ester: Fatty acids

Obese Zucker rats fed soya protein have a lower level of plasma cholesterol, accompanied by an increased faecal excretion of steroids when compared to those fed casein (Nagata *et al.* 1981, 1982; Tanaka *et al.* 1984; Madani *et al.* 1998; Wergedahl *et al.* 2004). Although the components and mechanisms responsible for the proposed cardioprotective effect of soya protein have not been clarified, several reports have shown that this might be due to components other than amino acids in soya products, e.g. isoflavones (primarily genistein and daidzein), which lower the plasma lipid levels (Huff *et al.* 1977; Potter, 1995; Balmir *et al.* 1996; Madani *et al.* 1998; Peluso *et al.* 2000; Fukui *et al.* 2002). The mechanisms of the cholesterol lowering of soya protein, however, are under some dispute.

The role of LDL receptors in the regulation of plasma cholesterol level is well established (Brown & Goldstein, 1986), and studies using a LDL receptor-deficient mouse model suggested that soya isoflavones may exert their effects by up-regulating LDL receptor activity (Kirk *et al.* 1998). Animal studies show that substituting soya protein for dietary animal protein reduces serum total and LDL-cholesterol concentrations (Sirtori *et al.* 1993), and it is suggested that the low ratios of methionine–glycine and lysine–arginine contribute to the cholesterol-lowering effect of soya protein (Kritchevsky *et al.* 1982; Morita *et al.* 1997).

An elevated plasma homocysteine level is recognised as a risk factor for cardiovascular disease (Nygard *et al.* 1997). Recently it has been demonstrated that soya protein has the capability to lower the plasma homocysteine in plasma of both type II diabetics and hypercholesterolaemic patients (Hermansen *et al.* 2001; Tonstad *et al.* 2002).

Zucker fa/fa fatty rats develop obesity with hyperphagia, severe hypertriglyceridaemia, mildly elevated plasma cholesterol level and high plasma levels of LDL and HDL (Bray, 1977), which makes them interesting as animal models in studies of obesity and diabetes II. The obese male Zucker fa/fa rats show little diurnal variation in the activities of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Lin, 1985) and cholesterol 7α hydroxylase (CYP7A1) in liver (Tang *et al.* 1988), and this makes them suitable for studying cholesterol metabolism. Although rats have no gall bladder, the Zucker fatty rat has considerably similarities to Type IV hyperlipoproteinaemia and can be used as an animal model for further studies on the relationship between faecal steroid excretion and cholesterol metabolism.

Intake of casein from cow's milk can cause hypercholesterolaemia and atheromatous plaques in animals due to its amino acid composition (Carroll & Hamilton, 1975). The question arises whether addition of a high level of isoflavones to a casein diet may prevent the unfavourable effects of casein when fed to obese Zucker rats. In the present study, a high dose of isoflavones (4·00 g genistein and 4·50 g daidzein per kg diet) was added to a casein diet HD1. Another group of rats was fed a single-cell protein-based diet (SCP), which has low methionine–glycine and lysine–arginine ratios, but is devoid of isoflavones, to see how this could affect cholesterol metabolism. To investigate how HDI and SCP affected cholesterol metabolism, the amounts of cholesterol and cholesteryl esters in liver and plasma of Zucker fa/fa rats were measured, and the fatty acid composition of the cholesteryl esters in liver was analysed. The faecal excretion of cholesterol and bile acids was measured, and the hepatic mRNA levels of LDL receptor, CYP7A1, sterol regulatory element binding protein (SREBP)-1a, SREBP-2, Liver X receptor (LXR) and LXRβ were quantified. The activities of the rate-determining enzymes in the biosynthesis of cholesterol (HMG-CoA reductase), cholesteryl esters (acyl-CoA:cholesterol acyltransferase, ACAT) and bile acids (CYP7A1) were also investigated to try to understand the mechanisms of action of diets with high content of isoflavones or with low ratios of methionine–glycine and lysine–arginine.

Materials and methods

Animals and diets

Two experiments were performed. In the first experiment, male Zucker rats Crl:(ZUC)/FaBR (fa/fa) from Charles River (Charles River Laboratory, Sulzfeld, Germany), weighing 80–135 g, were divided into three experimental groups of six rats each with comparable mean body weight. The rats were housed individually all through the feeding experiment, the first week of the experiment in metabolic cages, in a room maintained at a 12 h light–dark cycle and a constant temperature of 20 ± 3°C and relative humidity of 65 ± 15 %. The rats were acclimatised under these conditions before the start of the experiment. All rats had free access to tap water and food. Based on daily observations of the rats, it seemed that all experimental diets were well tolerated by the rats. The rats were fed diets consisting of 20 % (w/w) protein from fermented single-cell protein (Skrede *et al.* 1998), casein sodium salt from bovine milk (C-8654; Sigma-Aldrich Norway AS, Oslo, Norway), or a combination of casein and a fermented soya protein concentrate enriched in isoflavones for 6 weeks. Commercial soya oil (10 % w/w) was added to the diets. To all diets were added 1 % AIN-93VX Vitamin mixture (Dyets Inc., Bethlehem, PA, USA), 3 % AIN-93G MX Mineral mixture (Dyets Inc.) and 0·2 % choline hydrogentartrate (Merck, Darmstadt, Germany). HDI contained 4·00 g genistein and 4·50 g daidzein per kg diet. Table 1 gives an overview of the experimental diets.

In the second experiment, male Zucker rats Crl:(ZUC)/FaBR (fa/fa) from Charles River, weighing 80–135 g, were fed diets similar to the diets in the first experiment, except that they contained 20 % protein from either soya protein, fish protein hydrolysate (FPH) or casein, and the rats were fed these diets for 3 weeks. The soya protein and FPH diets had low methionine–glycine and

Table 1. Composition of the experimental diets (g/kg diet)†

	HDI‡	SCP§	Casein‖
Soya protein concentrate	67	–	–
Single cell protein	–	271	–
Casein	184	–	218
Soya oil¶	100	100	100
Sucrose	110	110	110
Vitamins††	10	10	10
Minerals‡‡	30	30	30
Cellulose	20	20	20
Dextrin	479	459	512

† The diets were isoenergetic and isonitrogenous, and contained 200 g crude protein/kg diet.

‡ HDI, diet containing a high dose of isoflavones (g/kg diet): fat (9·9), ash (30), genistein (4·0), daidzein (4·5). The fermented soya protein concentrate (enriched in isoflavones) contained 45·4 % crude protein.

§ SCP, single-cell protein-based diet (g/kg diet): fat (11·7), ash (38). The SCP protein contained 73·9 % crude protein.

‖ Casein (g/kg diet): fat (9·8), ash (31). The casein protein contained 91·9 % crude protein.

¶ Fatty acid composition of the soya oil (mean of two measurements, deviation < 3 %, shown as g/100 g fat): 18 : 2*n*-6 (55·9), 18 : 1*n*-9 (21·4), 16 : 0 (11·4), 18 : 3*n*-3 (5·8), 18 : 0 (3·3), 18 : 1*n*-7 (1·6).

†† AIN-93VX (Dyets Inc.).

‡‡ AIN-93GMX (Dyets Inc.).

lysine–arginine ratios, and are described in detail in Wergedahl *et al.* (2004).

At the end of the feeding period, the rats were anaesthetised with a 1:1 mixture of Hypnorm™ (fentanyl citrate 0·315 mg/ml and fluanisone 10 mg/ml; Janssen Animal Health, Janssen pharmaceutica, Beerse, Belgium) and Dormicum® (midazolam 5 mg/ml; F. Hoffmann-La Roche AG, Basel, Switzerland) injected subcutaneously. Blood was drawn directly from the heart using a syringe containing heparin. The liver was immediately removed, weighed and divided into two parts, which were immediately chilled on ice or frozen in liquid N_2. Plasma and liver were stored at −80°C until analysis. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Amino acids in diets and liver

The amino acids in the diets were determined after hydrolysis in 6 M-HCl at 110°C for 22 h and pre-derivatisation with phenylisothiocyanate according to the method of Cohen & Strydom (1988).

To analyse the amounts of free amino acids in liver, the samples were extracted and deproteinated by the addition of two volumes of 5 % sulphosalisylic acid, kept on ice for 30 min and centrifuged at 5000 *g* for 15 min. The supernatant was mixed with internal standard (norleucine).

The amino acids were quantified using a Biochrom 20 plus Amino Acid Analyzer (Amersham Pharmacia Biotech, Uppsala Sweden) equipped with a lithium column with post-column ninhydrin derivatisation as described by Liaset *et al.* (2003).

Cholesterol in plasma and liver

Plasma and liver cholesterol were measured enzymatically on the Technicon Axon system (Miles, Tarrytown, NY, USA) using the

following kits: total cholesterol (Bayer, Tarrytown, NY, USA), LDL-cholesterol (LDL-C plus from Roche Diagnostics, Mannheim, Germany), HDL-cholesterol (HDL-C plus from Roche Diagnostics) and free cholesterol (Wako Chemicals, Dalton, OH, USA). The amount of cholesteryl esters in plasma was calculated as the difference between total cholesterol and free cholesterol. Liver lipids were extracted by the method of Bligh & Dyer (1959), evaporated under N_2 and dissolved in isopropanol before analysis of total cholesterol (Bayer).

Fatty acid composition

Lipids were extracted from liver using a mixture of chloroform and methanol (Bligh & Dyer, 1959). The lipid classes in liver were separated by TLC on silica gel plates (0·25 mm silica gel 60; Merck) developed in hexane–diethyl ether–acetic acid (80:20:1, v/v.) (Mangold, 1969). Heneicosanoic acid (21:0) was added to the cholesteryl esters as internal standard. The extracts were transesterified using boron fluoride–methanol (Morrison & Smith, 1964). To remove neutral sterols and non-saponifiable material, the extracts were heated in 0·5 M-KOH in ethanol–water solution (9:1). Recovered fatty acids were re-esterified using boron fluoride–methanol. The methyl esters were quantified as previously described by Wergedahl *et al.* (2004). The concentration of cholesteryl esters in liver was calculated from the chromatograms as the sum of fatty acids esterified as cholesteryl esters.

Preparation of hepatic subcellular fractions

Homogenisation and subcellular fractionation of livers were performed as previously described by Berge *et al.* (1984). The procedure was performed at 0–4°C, and the fractions were stored at −80°C. Protein was assayed with the BioRad protein assay kit (BioRad, Richmond, CA, USA) using bovine serum albumin as the standard.

Enzyme assays

ACAT (*EC* 2.3.1.26; Field *et al.* 1991) and HMG-CoA reductase (*EC* 1.1.1.34; Brown *et al.* 1979) were measured in the hepatic microsomal fraction. Acyl-CoA synthetase (*EC* 6.2.1.3) was measured in the hepatic microsomal fraction by adding 20 µg protein to 250 µl assay mix (136 mM-Tris-HCl, 5·6 mM-MgCl$_2$ and 2·3 mM-EDTA, pH 7·4, 2·3 mM-ATP, 0·6 mM-CoA, 0·9 mM-dithiothreitol and 0·1 % Triton X-100), and the reaction was started with 25 µl 1·5 mM-[^{14}C]palmitic acid. The samples were incubated at 37°C for 3 min, before adding 1·05 ml 0·1 M-sodium acetate, pH 4·0 to stop the reaction. The product was extracted with addition of methanol–chloroform–heptane (1·41:1·25:1·00, v/v.), and the methanol-containing phase was counted.

Real-time quantitative RT-PCR

Total RNA was purified from frozen tissue using RNeasy Midi Kit (Qiagen, Hilden Germany). Primers and Taqman probes for rat ACAT-1, SREBP-1a and SREBP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) and the gene expression were determined using Taqman probes or SYBRgreen. The following sequences were used: ACAT-1 for-

ward 5′-AAA GAG GCG TGG GAT GCA-3′, reverse 5′-CAC GTC TGG TTT ACC TTT CAC TGA-3′ and probe 5′-TGC AAA TGA GAT TAC GCC CAT CAC CA-3′. SREBP-1a forward 5′-CGC CGC CGC CAT GGA CGA GC-3′ and reverse 5′-CAA ATA GGC CAG GGA AGT CAC-3′. SREBP-2 forward 5′-GGC CTG CCC CTA CCT ACT CTC-3′ and reverse 5′-TTT GTG GAC TGC TTG GCT CA-3′. GAPDH forward 5′-TGC ACC ACC AAC TGC TTA GC-3′, reverse 5′-CAG TCT TCT GAG TGG CAG TGA TG-3′ and probe 5′-TGG AAG GGC TCA TGA CCA CAG TCC A-3′.

LDL receptor, CYP7A1 (*EC* 1.14.13.17), LXRα and LXRβ are 'Assay-on Demand genes' designed by Applied Biosystems, with the following assay ID numbers: Rn00598438 (LDL receptor), Rn00564065 (CYP7A1), Rn00581185_m1 (LXRα) and Rn00581178_m1 (LXRβ). The probes and primers are a 20× mix, the final concentrations of the primers are 900 nM and that of the probes are 250 nM. Primers and Taqman probe for 18S rRNA were purchased from Applied Biosystems. Real-time PCR was carried out three times in triplicate for each sample on an ABI 7900 sequence detection system (Applied Biosystems). For each sample, results were normalised to GAPDH mRNA and 18S rRNA. Only results normalised to 18S rRNA are shown, as they were similar to the results normalised to GAPDH mRNA.

Faecal cholesterol and bile acid analysis

Faeces were collected from the first 7 d of the feeding experiment, and pooled for each rat. Faecal cholesterol was measured in lipids extracted from freeze-dried faeces by the method of Folch *et al.* (1957), washed in 0·88 % KCl evaporated under N_2 and dissolved in isopropanol before analysis. Total cholesterol was measured using the cholesterol kit from Bayer on the Technicon Axon system. Faecal total bile acids (3α-hydroxy bile acids) were measured in freeze-dried faeces by the method of Suckling *et al.* (1991), using Chromabond C18ec (3 ml/200 mg; Macherey-Nagel, Düren, Germany) and the enzymatic bile acid kit from Sigma Diagnostics (St Louis, MO, USA) on the Technicon Axon system.

Plasma homocysteine

Homocysteine in plasma was quantified by reverse-phase HPLC after derivatisation with the fluorescent agent monobromobimane, as described by Svardal *et al.* (1990).

Statistical analysis

All data in tables and figures are presented as mean values with their standard deviations for six rats per group. The data were evaluated by one-way ANOVA and Dunnett's test with the level of statistical significance set at $P < 0.05$ (GraphPad Prism version 3.0; GraphPad Prism, San Diego, CA, USA). Rats fed casein-based diet served as controls.

Results

Cholesterol metabolism

The total plasma cholesterol level was significantly reduced after feeding HDI and SCP compared to casein feeding
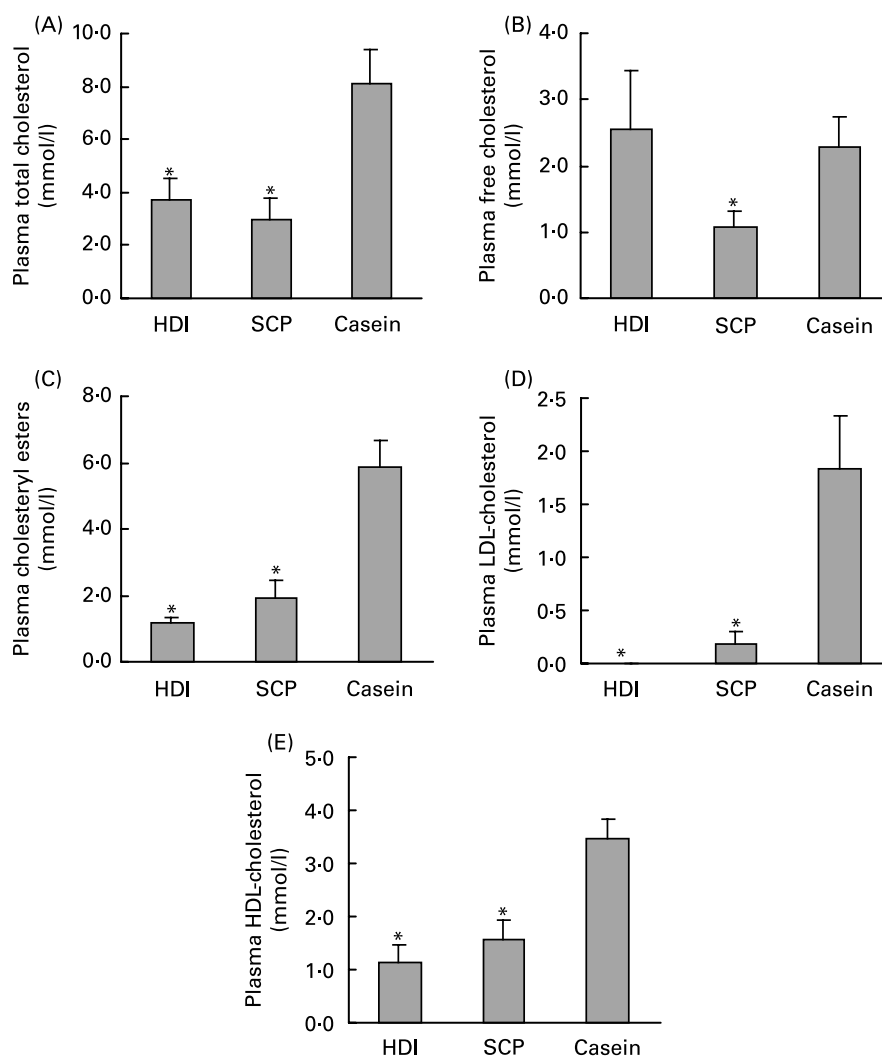
Fig. 1. Plasma cholesterol levels in obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), a single-cell protein-based diet (SCP) or casein. Values are means with standard deviations depicted by vertical bars for six rats per group. The figure shows the concentration of total cholesterol (A), free cholesterol (B), cholesteryl esters (C), LDL-cholesterol (D) and HDL-cholesterol (E). Mean values were significantly different from those of the control (casein) group: *$P < 0.05$.

(Fig. 1(A)). The SCP diet lowered the plasma levels of both free and esterified cholesterol (Fig. 1(B,C)), whereas HDI reduced only the concentration of cholesteryl esters (Fig. 1(C)). HDI and SCP feeding also reduced the LDL- and HDL-cholesterol levels in plasma (Fig. 1(D, E)).

HDI and SCP feeding reduced the hepatic concentrations of total cholesterol (Fig. 2(A)) and cholesteryl esters (Fig. 2(B)) when compared to the casein group. The mRNA level of the LDL receptor was almost doubled in the liver of rats fed HDI when compared to the casein group, whereas SCP had no effect (Fig. 2(C)). The microsomal activity of HMG-CoA reductase was increased after dietary administration of HDI or SCP (Fig. 3(A)), whereas the gene expression of SREBP-1a, SREBP-2 and LXRβ were unaffected by the diets (Table 2). However, a significant decrease was seen in the mRNA level of LXRα after HDI feeding when compared to the casein-fed rats (Table 2). The ACAT activity in liver was increased after HDI feeding, but not after SCP feeding, when compared to casein-fed rats (Fig. 3(B)). The gene expression of ACAT (ACAT-1) in liver was not significantly affected by any of the diets

(Table 2). However, the microsomal activity of acyl-CoA synthetase in liver was increased only after HDI feeding (Fig. 3(C)).

Both HDI and SCP feeding affected the fatty acid composition of cholesteryl esters in liver of obese Zucker rats (Table 3). The amount of $18:0$ was increased in the hepatic cholesteryl esters of Zucker rats fed HDI or SCP, whereas SCP feeding decreased the amount of $16:0$ when compared to casein (Table 3). HDI feeding increased the level of oleic acid ($18:1n$-9) whereas SCP feeding decreased the level of both $16:1n$-7 and oleic acid when compared to casein (Table 3). HDI feeding also decreased the total amount of n-6 PUFA, mainly due to decreased amounts of arachidonic acid ($20:4n$-6), but did not affect the total amount of n-3 PUFA, although the level of $18:3n$-3 and of $22:6n$-3 were markedly affected (Table 3). SCP feeding, on the other hand, increased the total level of both n-6 and n-3 PUFA, due to increased amounts of especially $18:2n$-6 and $20:5n$-3 (Table 3).

Faeces were collected during the first week of the feeding experiment and it was of interest that the amount of excreted bile acids in faeces was significantly increased after SCP feeding,
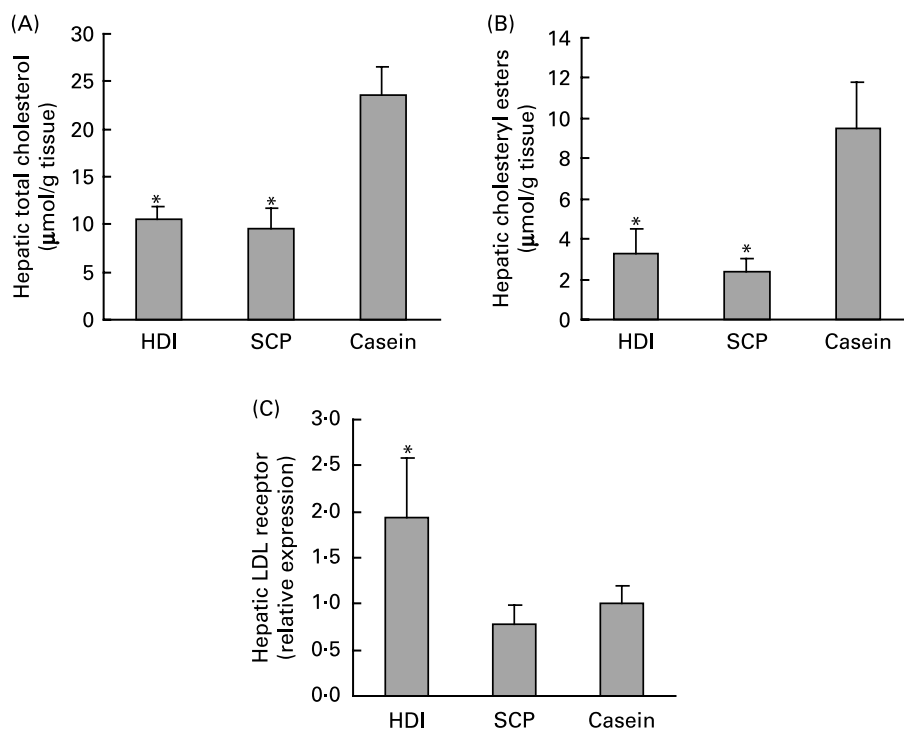
Fig. 2. Hepatic levels of total cholesterol and cholesteryl esters, and the gene expression of LDL receptor in obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), a single-cell protein-based diet (SCP) or casein. Values are means with standard deviations depicted by vertical bars for six rats per group. The figure shows the hepatic concentrations of total cholesterol (A) and cholesteryl esters (B), and mRNA level of LDL receptor (C). Mean values were significantly different from those of the control (casein) group: $*P < 0.05$.
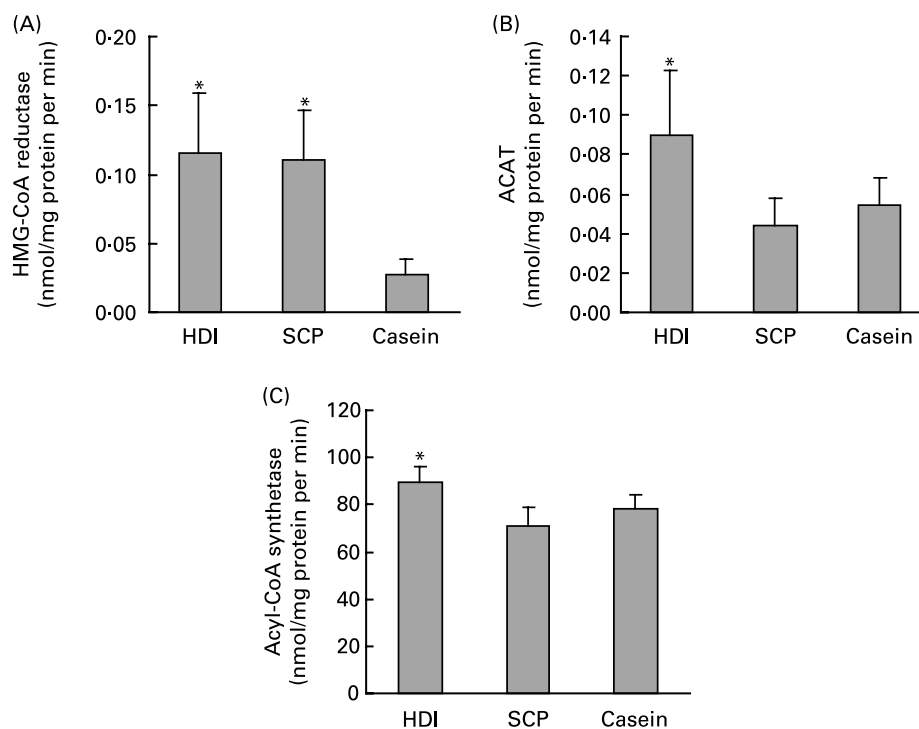
Fig. 3. Activities of enzymes involved in biosynthesis of cholesterol and cholesteryl esters in liver of obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), a single-cell protein-based diet (SCP) or casein. Values are means with standard deviations depicted by vertical bars for six rats per group. The figure shows the microsomal activities of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (A), acyl-CoA:cholesterol acyltransferase (ACAT; B) and acyl-CoA synthetase (C). Mean values were significantly different from those of the control (casein) group: $*P < 0.05$.

Table 2. mRNA levels (in arbitrary units) in liver of obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), single-cell protein-based diet (SCP) or casein†

(Mean values and standard deviations for six rats per group)

	HDI		SCP		Casein	
	Mean	SD	Mean	SD	Mean	SD
SREBP-1a	1·08	0·44	0·82	0·19	1·00	0·15
SREBP-2	0·77	0·33	1·15	0·18	1·00	0·16
LXRα	0·74*	0·16	0·92	0·11	1·00	0·07
LXRβ	0·75	0·29	0·90	0·16	1·00	0·12
ACAT-1	0·87	0·15	1·22	0·19	1·00	0·16

ACAT-1, acyl-CoA:cholesterol acyltransferase-1; LXRα, liver X receptor α; LXRβ, liver X receptor β; SREBP-1a, sterol regulatory element binding protein-1a; SREBP-2, sterol regulatory element binding protein-2.
Mean value was significantly different from that of the control (casein) group: *$P < 0.05$.
† For details of the diets and procedures, see Table 1 and pp. 322–323.

and tended to increase after HDI feeding when compared to casein-fed rats (Fig. 4(A)). Accordingly, a significant increase in the gene expression of CYP7A1 was observed in the liver of HDI- and SCP-fed rats (Fig. 4(B)). In addition, SCP increased the excretion of cholesterol in faeces more than fourfold when compared to casein-fed rats (Fig. 4(C)).

Amino acids

The methionine–glycine and lysine–arginine ratios were markedly lower in the SCP diet, due to a combination of higher levels of glycine and arginine, and lower levels of methionine and lysine when compared to the casein diet (Table 4). The HDI diet a consisted of 18·4 % casein and only 6·7 % of a fermented soya protein concentrate enriched in isoflavones, and the

Table 3. Selected fatty acids (g/100 g fatty acids) in hepatic cholesteryl esters of obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), single-cell protein-based diet (SCP) or casein†
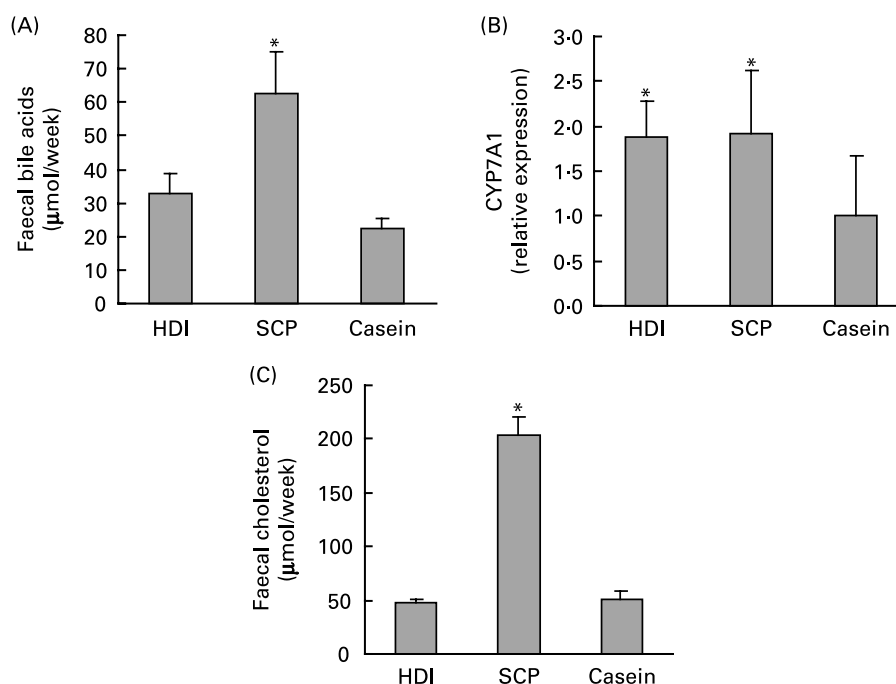
(Mean values and standard deviations for six rats per group)

	HDI		SCP		Casein	
	Mean	SD	Mean	SD	Mean	SD
16:0	22·3	1·2	18·8*	1·1	21·1	0·9
18:0	8·1*	2·5	8·3*	2·0	3·9	0·6
ΣSFA	30·4*	4·3	27·1	3·0	25·1	1·3
16:1*n*-7	13·2	1·5	6·7*	0·5	11·5	2·0
18:1*n*-9	35·7*	3·4	11·9*	1·2	28·1	5·0
ΣMUFA	48·7*	4·1	18·6*	1·4	39·7	6·6
18:2*n*-6	12·2	0·7	24·0*	3·0	11·9	1·4
18:3*n*-6	0·4	0·1	2·4*	0·3	0·7	0·2
20:3*n*-6	0·44	0·10	1·05*	0·06	0·36	0·03
20:4*n*-6	6·2*	1·3	24·3	1·9	20·6	6·1
Σ*n*-6 PUFA	19·2*	1·5	51·7*	3·0	33·6	7·5
18:3*n*-3	1·38*	0·18	0·51	0·10	0·51	0·05
20:5*n*-3	0·21	0·07	0·90*	0·17	0·26	0·06
22:5*n*-3	0·04	0·02	0·10*	0·03	0·06	0·01
22:6*n*-3	0·18*	0·02	1·02	0·37	0·81	0·18
Σ*n*-3 PUFA	1·57	0·68	2·53*	0·37	1·65	0·19

SFA, saturated fatty acids.
Mean values were significantly different from those of the control (casein) group: *$P < 0.05$.
† For details of the diets and procedures, see Table 1 and pp. 322–323.

methionine–glycine and lysine–arginine ratios are only marginally lower in HDI than in the casein diet (Table 4). However, both HDI and SCP feeding elevated the concentrations of both methionine and glycine in liver (Table 4). HDI feeding, but not SCP feeding, increased the hepatic level of lysine when compared to the casein group, whereas only trace amounts of arginine were found in the liver (Table 4). In plasma, HDI and SCP feeding reduced the homocysteine level (Fig. 5).

Effects of soya protein and fish protein hydrolysate in the diet

Feeding obese Zucker rats with soya protein or FPH reduced the total cholesterol level in liver when compared to casein-fed rats (Fig. 6(A)), but did not significantly change the gene expression of the LDL receptor (Fig. 6(B)). Soya protein feeding, but not FPH feeding, also led to an increase in the gene expression of CYP7A1 (Fig. 6(C)) and increased the faecal excretion of cholesterol (Fig. 6(D)).

Discussion

The hypocholesterolaemic effect of soya products is well documented, although there is some dispute over whether it is the amino acids or non-protein components, e.g. isoflavones, that are responsible for the cholesterol-lowering effect of soya (Huff *et al.* 1977; Potter, 1995; Balmir *et al.* 1996; Madani *et al.* 1998; Peluso *et al.* 2000; Fukui *et al.* 2002). Several studies have suggested that dietary proteins with low ratios of methionine–glycine and lysine–arginine, such as soya protein, have a cholesterol-lowering effect (Kritchevsky *et al.* 1982; Morita *et al.* 1997). In the present study we fed obese Zucker fa/fa rats with different diets containing either casein added a high level of isoflavones (HDI) or a protein with low ratios of methionine–glycine and lysine–arginine (SCP), resulting in reduced plasma levels of cholesterol when compared to casein-fed rats.

The methionine–glycine and lysine–arginine ratios were markedly lower in the SCP diet when compared to the casein diet, whereas only marginal changes in the ratios of methionine–glycine and lysine–arginine were seen in HDI relative to the casein diet (Table 4). As the amino acid profile of SCP, but not of HDI, favours a lowering of the plasma cholesterol (Kritchevsky *et al.* 1982; Morita *et al.* 1997), it is evident that some other component(s) must be responsible for the observed cholesterol-lowering effect of HDI. Although the amino acid composition was very different in SCP and HDI diets (Table 4), it was interesting that both diets led to similar changes in the hepatic concentrations of glycine and methionine when compared to the casein diet (Table 4).

Faecal excretion of neutral and acidic steroids is the major route of cholesterol removal from the body, and may be one mechanism to regulate the plasma cholesterol level. The faecal excretion of bile acids and cholesterol were increased several-fold in rats fed SCP when compared to the casein group (Fig. 4(A, C)), accompanied by an increased hepatic mRNA level of CYP7A1 (Fig. 4(B)). The increased faecal removal of steroids from the liver of SCP-fed rats probably caused the observed depletion of hepatic cholesterol (Fig. 2(A)) and the decreased levels of total cholesterol, free cholesterol, cholesteryl esters, LDL-cholesterol and HDL-cholesterol in plasma (Fig. 1). On the other hand, HDI feeding increased the gene expression of CYP7A1 (Fig. 4(B)) but had only a minor effect on the bile acid level and no effect on the cholesterol level in faeces

Fig. 4. Faecal excretion of bile acids and cholesterol during the first week of feeding, and the hepatic gene expression of cholesterol 7α hydroxylase (CYP7A1) in obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), a single-cell protein-based diet (SCP) or casein. Values are means with standard deviations depicted by vertical bars for six rats per group. The figure shows the faecal excretion of bile acids (A), the mRNA level of CYP7A1 (B) and the faecal excretion of cholesterol (C). Mean values were significantly different from those of the control (casein) group: *$P < 0.05$.

(Fig. 4(A, C)), indicating that the reabsorption of bile was higher in rats fed HDI than in those fed casein. In a different experiment, obese Zucker rats were fed soya protein and FPH, both with low ratios of methionine–glycine and lysine–arginine when compared to casein (Wergedahl *et al.* 2004). Interestingly, although both soya protein and FPH lowered the cholesterol level in plasma (Wergedahl *et al.* 2004) and in liver (Fig. 6(A)) when compared to casein, only soy protein increased the faecal excretion of both bile acids (Wergedahl *et al.* 2004) and choles-

terol (Fig. 6(D)), followed by an enhanced gene expression of CYP7A1 (Fig. 6(C)). These observations suggest that the mechanism of action for the cholesterol-lowering effect of SCP and soya protein seemed to be mediated, at least partly, via the enterohepatic pathway while HDI and FPH affected the cholesterol level via other mechanisms.

HDI feeding, but not SCP, soya protein or FPH feeding caused a marked increase in the gene expression of LDL receptor in liver when compared to casein feeding (Fig. 2(C)). The LDL receptor

Table 4. Amino acids in the experimental diets and in liver of obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), single-cell protein-based diet (SCP) or casein†

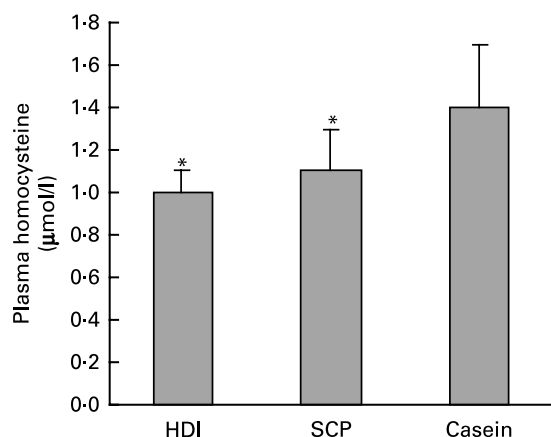|	HDI		SCP		Casein	
Diets (g/kg)‡						
Arginine	9·3		13·7		8·3	
Glycine	4·5		9·8		4·1	
L-Lysine	15·7		11·3		16·9	
L-Methionine	3·6		2·6		4·5	
Amino acid ratios						
L-Methionine–glycine	0·8		0·3		1·1	
L-Lysine–arginine	1·7		0·8		2·0	
Liver§						
μmol/g liver	Mean	SD	Mean	SD	Mean	SD
Arginine	Trace‖		Trace		Trace	
Glycine	1·8*	0·3	1·7*	0·1	1·0	0·1
L-Lysine	1·8*	0·2	1·1	0·1	1·2	0·1
L-Methionine	0·41*	0·04	0·27*	0·02	0·18	0·04

Mean values were significantly different from those of the control (casein) group: *$P < 0.05$.
† For details of the diets and procedures, see Table 1 and pp. 322–323.
‡ Mean values of two measurements. The difference between the two measurements was <3 %.
§ Mean values and standard deviations for six rats per group.
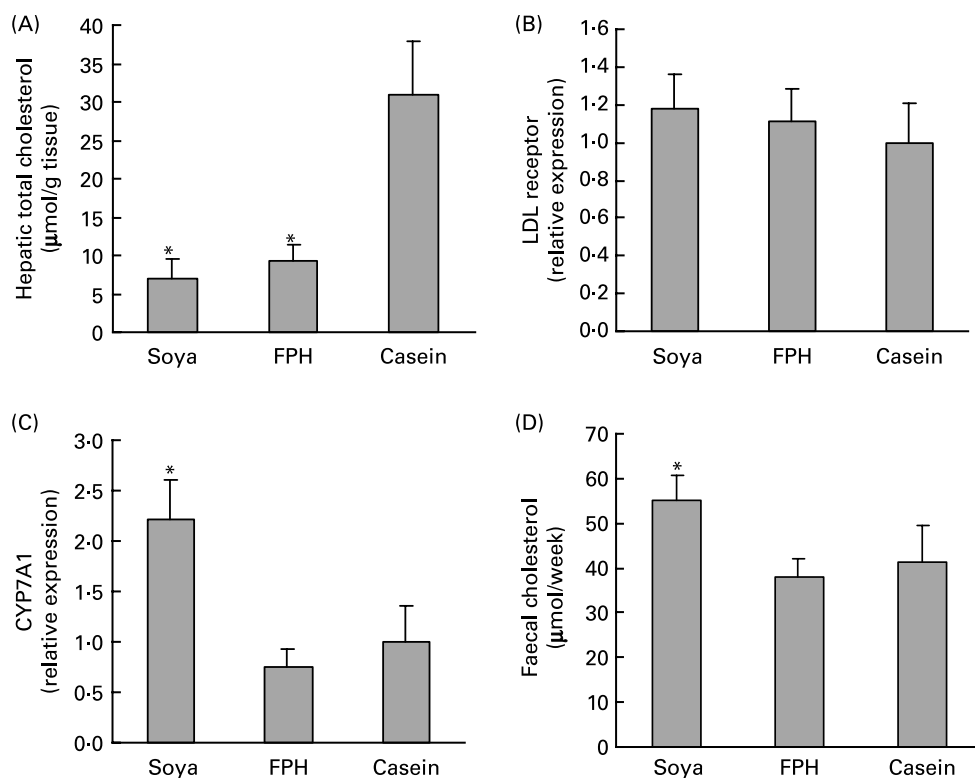‖ Trace, <0·03 μmol/g liver.

Fig. 5. Plasma homocysteine levels in obese Zucker rats fed a diet containing a high dose of isoflavones (HDI), single-cell protein-based diet (SCP) or casein. Values are means with standard deviations depicted by vertical bars for six rats per group. Mean values were significantly different from those of the control (casein) group: *$P < 0.05$.

gene expression (Fig. 2(C)), which will enhance the movement of cholesterol towards the liver from the circulation. Our data are in accordance with Kirk et al. (1998) who suggested that isoflavones lower the cholesterol level by increasing the LDL receptor activity. Thus the effect of HDI on the LDL receptor gene expression suggests that HDI exerts its cholesterol-lowering effect via the circulation.

Several transcription factors are involved in the regulation of cholesterol metabolism. LXRα and LXRβ regulate excess free cholesterol in cells by activating genes that control the rate of bile acid synthesis and cholesterol efflux (Lehmann et al. 1997), and SREBP-1a and SREBP-2 are involved in the regulation of cholesterol biosynthesis (Horton et al. 1998). In spite of up-regulated levels of the CYP7A1 mRNA (Fig. 4(B)) and increased HMG-CoA reductase activity (Fig. 3(A)), the gene expressions of LXRα, LXRβ, SREBP-1a and SREBP-2 were not enhances by HDI and SCP feeding (Table 2). This indicated that an increase of these transcription factors was not necessary to increased excretion of bile acids or increased cholesterol biosynthesis. The increased activity of HMG-CoA reductase activity observed after HDI and SCP feeding was possibly compensatory for the reduced cholesterol level in liver.

As the enterohepatic circulation seemed to be of minor importance for the cholesterol-lowering effect of HDI (Fig. 4), it is possible that an appreciable amount of cholesterol instead is esterified as the activity of ACAT is increase (Fig. 3(B)). ACAT esterifies excess free cholesterol with fatty acyl-CoA to form cholesteryl esters for either storing or secretion as lipoproteins, and the activity of ACAT depends on substrate availability, i.e. choles-

binds apo B-100, the sole protein of LDL (Goldstein & Brown, 1974) and apoE, found in HDL (Bersot et al. 1976; Innerarity & Mahley, 1978), and lipoproteins that contain multiple copies of apoE, as is the case of HDL in obese Zucker rats (Blay et al. 2001), binds with high affinity to LDL receptors (Innerarity & Mahley, 1978). The reduced plasma levels of LDL-cholesterol (Fig. 1(D)) and HDL-cholesterol (Fig. 1(E)) seen after HDI feeding could therefore be due to the increased hepatic LDL receptor

Fig. 6. Cholesterol level and gene expression of LDL receptor and cholesterol 7α hydroxylase (CYP7A1) in liver, and cholesterol level in faeces of obese Zucker rats fed soya protein (soy), fish protein hydrolysate (FPH) or casein. Values are means with standard deviations depicted by vertical bars for six rats per group. The figure shows the hepatic concentration of total cholesterol (A), mRNA level of LDL receptor (B), mRNA level of CYP7A1 (C) and the faecal excretion of cholesterol (D). Mean values were significantly different from those of the control (casein) group: *$P < 0.05$.

terol and acyl-CoA, in the liver. The increased activities of ACAT (Fig. 3(B)) and acyl-CoA synthetase (Fig. 3(C)), catalysing the production of acyl-CoA, suggested that esterification of cholesterol might be important for the cholesterol-lowering effect of HDI. No significant changes were seen in the gene expression of ACAT-1 (Table 2), indicating that the regulation of the ACAT activity was not at the transcriptional level.

Oleic acid is the preferred substrate for ACAT, and concomitant with an elevated ACAT activity in HDI-fed rats (Fig. 3(B)), the hepatic cholesteryl esters contained increased amounts of oleic acid. The marked decrease in the level of arachidonic acid implied that there was an inhibition of the flux of fatty acids towards arachidonic acid after HDI feeding. In SCP-fed rats, however, n-3 and n-6 PUFA dominated at the expense of $16:1n$-7 and oleic acid in the cholesteryl esters (Table 3). Thus, even though both HDI and SCP feeding lowered the hepatic level of cholesteryl esters (Fig. 2(B)), the fatty acid composition of cholesteryl esters were affected differently by these diets (Table 3).

High plasma level of homocysteine is associated with increased risk of various CVD including CHD (Nygard *et al.* 1997). Therefore, it was of interest that the concentration of homocysteine was decreased in plasma of rats fed HDI SCP (Fig. 5). Reduced plasma level of homocysteine concomitant with reduced amounts of cholesterol and especially LDL-cholesterol in plasma suggests HDI and SCP as potentially cardioprotective agents.

In summary, the experiments showed that several mechanisms for the cholesterol-lowering effect of dietary proteins probably exist. Our observations suggest that the mechanism of action for the cholesterol-lowering effect of SCP seemed to be mediated via the enterohepatic circulation, whereas HDI seemed to lower the plasma cholesterol level via circulation. Diets enriched in isoflavones and diets containing proteins with low methionine–glycine and lysine–arginine ratios lowered the plasma cholesterol and homocysteine levels, favouring a cardioprotective effect.

Acknowledgements

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