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Dietary soya protein concentrate enriched with isoflavones reduced fatty liver, increased hepatic fatty acid oxidation and decreased the hepatic mRNA level of VLDL receptor in obese Zucker rats

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Casein-based diets containing a low (LDI) or high (HDI) dose of soya protein concentrate enriched with isoflavones were fed to obese Zucker rats for 6 weeks. HDI feeding, but not LDI feeding, reduced the fatty liver and decreased the plasma levels of alanine transaminase and aspartate transaminase. This was accompanied by increased activities of mitochondrial and peroxisomal β-oxidation, acetyl-CoA carboxylase, fatty acid synthase and glycerol-3-phosphate acyltransferase in liver and increased triacylglycerol level in plasma. The decreased fatty liver and the increased plasma triacylglycerol level appeared not to be caused by an increased secretion of VLDL, as HDI decreased the hepatic mRNA levels of apo B and arylacetamide deacetylase. However, the gene expression of VLDL receptor was markedly decreased in liver, but unchanged in epididymal white adipose tissue and skeletal muscle of rats fed HDI, indicating that the liver may be the key organ for the reduced clearance of triacylglycerol-rich lipoproteins from plasma after HDI feeding. The *n*-3/*n*-6, 20 : 4*n*-6/18 : 2*n*-6 and (20 : 5*n*-3 + 22 : 6*n*-3)/18 : 3*n*-3 ratios were increased in liver triacylglycerol by HDI. The phospholipids in liver of rats fed HDI contained a low level of 20 : 4*n*-6 and a high level of 20 : 5*n*-3, favouring the production of anti-inflammatory eicosanoids. When obese Zucker rats were fed soya protein, this also resulted in reduced fatty liver, possibly through reduced clearance of VLDL by the liver. We conclude that the isoflavone-enriched soya concentrate as well as soya protein may be promising dietary supplements for treatment of non-alcoholic fatty liver.

Steatosis: β-Oxidation: Lipogenesis

Non-alcoholic fatty liver disease affects 10–24 % of the general global population, but the prevalence increases significantly, to more than 50 %, for obese people (Silverman *et al.* 1990; Angulo & Lindor, 2002). It has been estimated that 5–10 % of obese individuals are obese due to insufficient leptin production, while the remaining 90–95 % are believed to be leptin resistant (Cohen & Friedman, 2004). The genetically obese Zucker fa/fa rats have a null mutation of the leptin receptor gene and have been extensively studied as a model for obesity in man. These rats also have high plasma triacylglycerol levels and abnormally high synthesis of triacylglycerol in liver, combined with a low rate of fatty acid oxidation in liver (Bray, 1977; Triscari *et al.* 1982), leading to development of fatty liver at a young age (Krief & Bazin, 1991). The obese Zucker fa/fa rats may therefore be useful as an animal model for a deeper understanding of biochemical changes that occur during the development and treatment of fatty liver.

There is no widely accepted therapy for fatty liver, but lifestyle modifications with weight reduction are frequently recommended (McClain *et al.* 2004), and one approach has been changes in the diet with special focus on dietary fats. Increasing evidence suggests that not only dietary lipids and fatty acids, but also proteins and amino acids in the diet can affect lipid metabolism. It has been known for some years that dietary soya products lower the serum total cholesterol and LDL cholesterol in man (Anderson *et al.* 1995) and animals (Sirtori *et al.* 1993), and may have a cardioprotective effect. The components and mechanisms responsible for the hypocholesterolaemic effect of soya products have not been clarified. It has been suggested that the amino acid composition of soya protein, with low ratios of methionine/glycine and lysine/arginine, contribute to its cholesterol-lowering effect (Kritchevsky *et al.* 1982; Morita *et al.* 1997), but several reports have shown that this might be due to components other than amino acids, e.g. isoflavones (primarily genistein and daidzein) that are associated with the soya protein (Huff *et al.* 1977; Potter, 1995; Balmir *et al.* 1996; Madani *et al.* 1998; Peluso *et al.* 2000; Fukui *et al.* 2002; Ali *et al.* 2004).

Abbreviations: AADA, arylacetamide deacetylase; CPT, carnitine palmitoyltransferase; FAT/CD36, fatty acid translocase; HDI, diet containing a high dose of soya protein concentrate enriched with isoflavones; LDI, diet containing a low dose of soya protein concentrate enriched with isoflavones; LPL, lipoprotein lipase; SCD-1, stearoyl-CoA desaturase; WAT, white adipose tissue.

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We have previously demonstrated that when obese Zucker rats were fed a diet containing soya protein, which contains isoflavones, a lowering of the plasma cholesterol level and of the lipid content in liver resulted (Wergedahl *et al.* 2004). Similar results have also been reported by Peluso *et al.* (2000) after dietary treatment with the isoflavone-enriched fraction of isolated soya protein to obese Zucker rats. In the present study we performed two experiments: first, we wanted to study whether a dietary soya concentrate enriched in isoflavones could reduce the fatty liver in obese Zucker rats, and to elucidate the mechanism behind this event; second, obese Zucker rats were fed intact soya protein to see whether this would reduce the fatty liver via the same mechanisms as the isoflavone-enriched soya concentrate.

Materials and methods

Animals and diets

Two experiments were performed. In the first experiment, male obese Zucker rats Crl:(ZUC)/FaBR (fa/fa) from 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 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. The rats were fed diets consisting of 20 % (by weight) protein from casein sodium salt from bovine milk (C-8654; Sigma-Aldrich Norway AS, Oslo, Norway) or casein-added fermented soyabeans with high contents of genistein and daidzein. Two doses of isoflavones were used. The diet with a low amount of soya protein concentrate enriched with isoflavone (LDI) contained 21·4 % casein and 0·7 % fermented soya proteins, providing 0·40 g genistein/kg diet and 0·45 g daidzein/kg diet. The diet with a high-dose of soya protein concentrate enriched with isoflavone (HDI) contained 18·4 % casein and 6·7 % fermented soya proteins, providing 4·00 g genistein/kg diet and 4·50 g daidzein/kg diet. Choline hydrogentartrate (0·2 %; Merck, Darmstadt, Germany) was added to all diets. The rats were fed these diets for 6 weeks. All rats had free access to tap water and feed. Based on daily observations of the rats, it seemed that all experimental diets were well tolerated. Table 1 gives an overview of the experimental diets.

In the second experiment, male obese Zucker rats Crl:(ZUC)/FaBR (fa/fa) from Charles River Laboratory, 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 or casein, and the rats were fed these diets for 3 weeks. Table 1 gives an overview of the experimental diets (for more details on the diets and experimental conditions, see Wergedahl *et al.* 2004).

At the end of the feeding period, under non-fasting conditions, the rats were anaesthetised with a 1:1 mixture of Hypnorm™ (0·315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen Pharmaceutica, Beerse, Belgium) and Dormicum® (5 mg/ml midazolam; 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. Plasma, liver, epididymal white adipose tissue (WAT) and skeletal muscle from the thigh were stored at −80° until analysis. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

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

	Experiment 1			Experiment 2	
	Casein†	LDI‡	HDI§	Casein†	Soya‖
Casein sodium salt	218	214	184	218	–
Soya protein concentrate	–	7	67	–	–
Soya protein	–	–	–	–	227
Soya oil¶	100	100	100	100	100
Sucrose	110	110	110	110	110
Vitamins**	10	10	10	10	10
Minerals††	30	30	30	30	30
Cellulose	20	20	20	20	20
Dextrin	512	509	479	491	484

* The diets were isoenergetic and isonitrogenous, and contained 200 g crude protein/kg diet.
† Casein (g/kg diet): fat (98), ash (31). The casein protein contained 91·9 % crude protein.
‡ LDI, diet containing a low dose of soya protein concentrate enriched with isoflavones (g/kg diet): fat (99), ash (31), genistein (0·40), daidzein (0·45). The soya protein concentrate contained 45·4 % crude protein
§ HDI, diet containing a high dose of soya protein concentrate enriched with isoflavones (g/kg diet): fat (99), ash (30), genistein (4·00), daidzein (4·50). The soya protein concentrate contained 45·4 % crude protein.
‖ Diet containing soya protein (g/kg diet): fat (101), ash (54). The soya protein contained 88·0 % crude protein. The diets in Experiment 2 had 2 % NaCl added.
¶ Fatty acid composition of the soya oil (mean of two measurements, deviation less than 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., Bethlehem, PA, USA).
†† AIN-93G-MX (Dyets Inc.).

Fatty acid composition

Lipids were extracted from liver and plasma samples 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, by vol.; Mangold, 1969). The spots were identified using Rhodamine G (Fluka Chemie AG, Buchs, Switzerland) and co-migration with known standards. The monoacylglycerol and phospholipid spots were incompletely separated and were examined as one fraction, referred to as the phospholipid fraction. Heneicosanoic acid (21 : 0) was added to the extracts 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 (Wergedahl *et al.* 2004).

Scharlach red staining of liver

In order to study neutral fat deposits, 10 μm frozen sections from the livers were cut, stained with a filtered Scharlach red solution and visualised by light microscopy.

Lipid quantification

Triacylglycerol in liver, plasma and triacylglycerol-rich lipoproteins were measured enzymatically using the triacylglycerol kit from Bayer (Tarrytown, NY, USA). Phospholipids in liver were measured using the phospholipid kit from bioMerieux (Lyon, France). NEFA in plasma were measured using the NEFA C kit from Wako Chemicals (Dalton, OH, USA) on the Hitachi 917 system (Roche Diagnostics GmbH, Mannheim, Germany). Liver lipids were extracted by the method of Bligh and Dyer (Bligh & Dyer, 1959), evaporated under N and re-dissolved in isopropanol before analysis.

Plasma transaminases

The plasma levels of alanine transaminase and aspartate transaminase were measured on the Hitachi 917 system (Roche Diagnostics) using the appropriate kits from Roche Diagnostics.

Real-time quantitative RT–PCR

Total RNA was purified from frozen liver, epididymal WAT and skeletal muscle using RNeasy Midi Kit (Qiagen, Hilden, Germany). For isolation of RNA from WAT, QIAzol Lysis Reagent (Qiagen) was added to the samples and the extraction was performed with chloroform. For isolation of RNA from muscle, proteinase K (Qiagen) was added to the samples. Primers and Taqman probe for rat carnitine palmitoyltransferase (CPT)-Ia, CPT-II, Δ5 desaturase, Δ6 desaturase, glyceraldehyde-3-phosphate dehydrogenase, PPARα, stearoyl-CoA desaturase and sterol regulatory binding protein-1c were designed using Primer Express (Applied Biosystems, Foster City, CA, USA). Gene expressions were determined using Taqman probes or SYBRgreen. The following sequences were used: CPT-Ia forward 5′-CCC AGT GGG AGC GAC TCT T-3′, reverse 5′-TGT GCC TGC TGT CCT TGA TAT G-3′ and probe 5′-AGG AGA CAG ACA CCA TC-3′. CPT-II forward 5′-ATT ATC TGC AGC ACA GCA TCG T-3′, reverse 5′-TGC ATT GAG GTA TCT CTT CAT GGT-3′ and probe 5′-TGC CCA GGC TGC CTA-3′. Δ5 desaturase forward 5′-TGG ATC TTT GGA ACT TCC TTG GT-3′, reverse 5′-CAA AGT CAT GCT GTA GCC AAC CT-3′ and probe 5′-CAG TTC AGG CCC AGG C-3′. Δ6 desaturase forward 5′-CAG CGG GCA CCT CAA TTT-3′, reverse 5′-TGC TTG GCG CAG AGA GAC T-3′ and probe 5′-CAG ATT GAG CAC CAC CTC TTC CCC AC-3′. Glyceraldehyde-3-phosphate dehydrogenase 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′. PPARα forward 5′-TGG AGT CCA CGC ATG TGA AG-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′. Stearoyl-CoA desaturase-1 forward 5′-CCT CAT CAT TGC CAA CAC CAT-3′, reverse 5′-CGG CGT GTG TCT CAG AGA AC-3′ and probe 5′-TCC CAG AAC GAT GTA TAT GAA TGG GCC C-3′. Sterol regulatory element binding protein-1c forward 5′-GGA GCC ATG GAT TGC ACA TTT G-3′, reverse 5′-CAA ATA GGC CAG GGA AGT CAC-3′. apo B (Rn01499049_g1), arylacetamide deacetylase (AADA; Rn00571934_m1), elongase (Rn00592812), fatty acid translocase (FAT/CD36; Rn00580728_m1), hormone-sensitive lipase (Rn00563444_m1), liver fatty acid-binding protein (Rn00664587_m1), lipoprotein lipase (LPL; Rn00561482_m1), PPARδ (Rn00565707_m1), PPARγ (Rn00440945_m1) and VLDL receptor (Rn005784) are 'Assay-on Demand' designed by Applied Biosystems.

Real-time RT–PCR was carried out in triplicate on an ABI 7900 sequence detection system (Applied Biosystems). A dilution curve from one cDNA source using dilutions 1:2, 1:4, 1:8 and a no-template control was run for each gene. The gene expression was determined by relative quantification using the standard curve method. For each sample, results were normalised to glyceraldehyde-3-phosphate dehydrogenase mRNA and 18S rRNA (RT-CKFT-18S; MedProbe, Oslo, Norway). Only results normalised to 18S rRNA are shown, as they were similar to the results normalised to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Preparation of hepatic subcellular fractions

Homogenisation and subcellular fractionation of the livers were performed as previously described (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

Palmitoyl-CoA and palmitoyl-L-carnitine oxidation were measured in the mitochondrial fraction as acid-soluble products (Willumsen *et al.* 1993). CPT-I and CPT-II activities were measured in the mitochondrial fraction (Madsen *et al.* 1998). Fatty acyl-CoA oxidase was measured in the peroxisomal fraction (Small *et al.* 1985). 3-Hydroxy-3-methylglutaryl-CoA synthase was measured spectrophotometrically in the mitochondrial fraction (Clinkenbeard *et al.* 1975). Acetyl-CoA carboxylase activity was measured in cytosolic fraction by measuring the amount of $NaH^{14}CO_3$ incorporated into malonyl-CoA (Tanabe *et al.* 1981). Fatty acid synthase activity was measured in the cytosolic fraction as described by Roncari (1981), modified according to Skorve *et al.* (1993). Glycerol-3-phosphate acyl transferase activity was measured in mitochondrial and microsomal fractions as described by Bates & Saggerson (1977). Acyl-CoA synthetase activity was measured in the mitochondrial fraction (Gudbrandsen *et al.* 2005).

Malonyl-CoA

Malonyl-CoA in the liver was measured by reversed-phase HPLC (Wergedahl *et al.* 2004).

Isolation of triacylglycerol-rich lipoproteins

Plasma from two rats was pooled to obtain a volume of 3 ml. The plasma triacylglycerol-rich lipoprotein fraction was prepared as previously described (Muna *et al.* 2002).

Statistical analysis

All data in the tables are presented as means and standard deviations for six rats per group. The data were evaluated by one-way ANOVA and Dunnett's test (Experiment 1, when three feeding groups were included) or by unpaired Student's *t* test (Experiment 1, when two feeding groups were included, and Experiment 2) with the level of statistical significance set at $P<0{\cdot}05$ (GraphPad Prism, version 3.0; GraphPad Prism, San Diego, CA, USA). Rats fed casein-based diet served as controls.

Results

Hepatic lipids and fatty acid composition

Scharlach red staining of the livers demonstrated that the levels of neutral lipids were decreased by HDI feeding, as the lipid droplets were both fewer and smaller (Fig. 1). Quantification of the hepatic triacylglycerol level showed that HDI reduced the level of triacylglycerol by 67 % (Table 2). The reduced fatty liver by HDI was accompanied with reduced plasma levels of alanine transaminase and aspartate transaminase by more than 80 % (Table 2). The hepatic phospholipid level was not changed after HDI feeding (Table 2). When rats were fed LDI, no effects were seen in the liver lipid content or in the plasma transaminases (Fig. 1; Table 2).

HDI feeding changed the fatty acid composition of the hepatic lipids (Table 3). The level of 16 : 0 was decreased in triacylglycerol, phospholipids and 1,2-diacylglycerol, whereas the level of 18 : 0 was increased in phospholipids and 1,2-diacylglycerol after HDI feeding (Table 2). This resulted in an increased 18 : 0/16 : 0 ratio in phospholipids, triacylglycerol and 1,2-diacylglycerol (calculated from Table 3), concomitant with an increased mRNA level of elongase in liver of HDI-fed rats (Table 4).

The levels of the Δ9 desaturated fatty acids 16 : 1*n*-7 and 18 : 1*n*-9 in liver lipids were not changed by HDI feeding, except for a decreased level of 18 : 1*n*-9 in 1,2-diacylglycerol (Table 3). The mRNA level of stearoyl-CoA desaturase-1, the rate-limiting enzyme catalysing the synthesis of Δ9 desaturated MUFA, was not significantly changed by HDI (Table 4).

The phospholipid levels of 18 : 2*n*-6, 18 : 3*n*-6, 20 : 3*n*-6, 18 : 3*n*-3 and 20 : 5*n*-3 were increased and the level of 20 : 4*n*-6 was decreased after HDI feeding. In addition, HDI-fed rats had an increased level of 20 : 3*n*-6 in triacylglycerol and 1,2-diacylglycerol and of 20 : 4*n*-6 acid in triacylglycerol, 1,2-diacylglycerol and as NEFA. HDI feeding also increased the level of 20 : 5*n*-3 in 1,2-diacylglycerol, and that of 22 : 5*n*-3 and 22 : 6*n*-3 in triacylglycerol (Table 3). The ratio of *n*-3 to *n*-6 was increased in triacylglycerol and decreased in 1,2-diacylglycerol by HDI feeding (Table 5). HDI feeding decreased the 20 : 4*n*-6/18 : 2*n*-6 and the (20 : 5*n*-3 + 22 : 6*n*-3)/18 : 3*n*-3 ratios in phospholipids, whereas these ratios were increased in triacylglycerol (Table 5). The Δ6 and Δ5 desaturases catalyse the biosynthesis of long-chain *n*-6 and *n*-3 PUFA from the precursors 18 : 2*n*-6 and 18 : 3*n*-3, respectively. The mRNA level of Δ6 desaturase was not affected, but the mRNA level of Δ5 desaturase was significantly decreased by HDI feeding (Table 4).

The fatty acid composition of phospholipids, triacylglycerol, 1,2-diacylglycerol and NEFA and the mRNA levels of elongase and desaturases in liver were not affected by LDI feeding (data not shown).

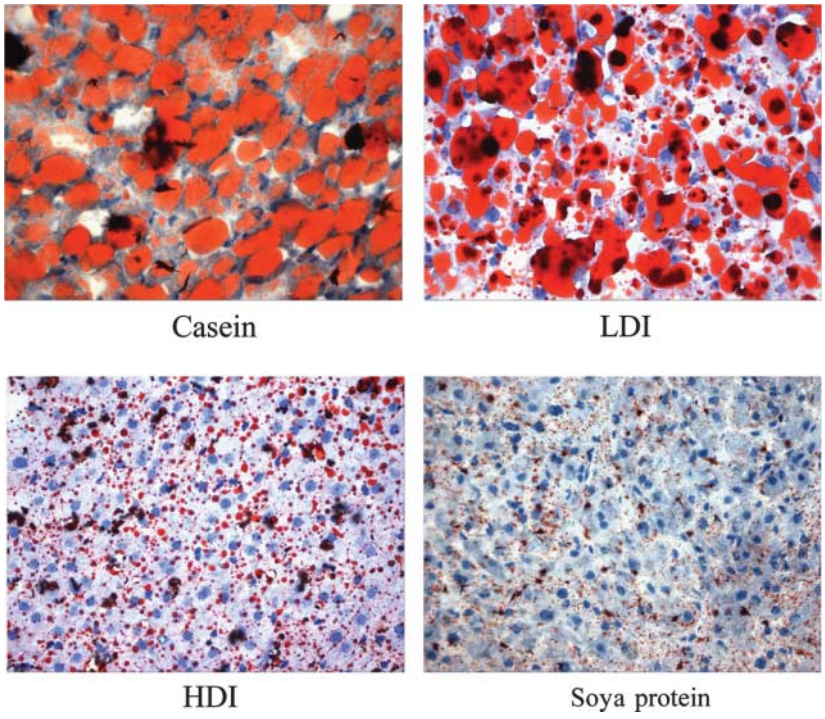

Fig. 1. Fat-stained microphotographs from livers of Zucker rats fed a diet containing casein, a low dose of soya protein concentrate enriched with isoflavones (LDI), a high dose of soya protein concentrate enriched with isoflavones (HDI) or soya protein, showing one representative example from each feeding group. For details of procedures, see p. 250. Scharlach red, original magnifications × 50.

Table 2. Hepatic lipids and plasma transaminases in obese Zucker rats fed a diet containing casein, a low dose of soya protein concentrate enriched with isoflavones (LDI) or a high dose of soya protein concentrate enriched with isoflavones (HDI)†

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

	Casein		LDI		HDI	
	Mean	SD	Mean	SD	Mean	SD
Hepatic lipids (μmol/g liver)						
Triacylglycerol	404	59	373	59	135*	20
Phospholipids	45	5	44	5	43	5
Plasma transaminases (U/l)						
Alanine transaminase	465	155	417	269	83*	24
Aspartate transaminase	973	270	569	235	128*	31

Mean values were significantly different from those of the control (casein) group: *$P<0{\cdot}05$.

† For details of diets and procedures, see Table 1 and p. 250.

Hepatic lipid metabolism

The activation of fatty acids in mitochondria was increased, as the activity of mitochondrial acyl-CoA synthetase was increased by 69 % after HDI feeding (Table 6). HDI feeding increased the mitochondrial β-oxidation, measured as acid-soluble products after oxidation of palmitoyl-CoA and palmitoyl-L-carnitine by 59 and 110 %, respectively (Table 6). This was accompanied by a 55 % increase in the CPT-II activity, whereas the CPT-I activity was similar to that of controls (Table 6). However, the gene expressions of CPT-I and CPT-II were not affected by HDI feeding (Table 4). The malonyl-CoA level and the malonyl-CoA sensitivity of CPT-I in liver of rats fed HDI were similar to controls (data not shown). The peroxisomal β-oxidation, measured as acyl-CoA oxidase activity in peroxisomes, was increased by 52 % after HDI feeding (Table 6). The gene expressions of PPARα and PPARδ in liver were decreased by 47 and 36 % after HDI feeding, respectively, whereas the mRNA level of liver fatty acid-binding protein was unchanged (Table 4). The activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, the rate-limiting enzyme in ketogenesis, was not affected by HDI feeding (Table 6).

HDI feeding affected the biosynthesis of both fatty acids and glycerolipids, as the cytosolic activities of acetyl-CoA carboxylase and fatty acid synthase as well as mitochondrial and microsomal glycerol-3-phosphate acyltransferase were increased by 86, 170, 175 and 52 %, respectively (Table 6). In addition, the gene expression of sterol regulatory element binding protein-1c was increased by 138 %, whereas the mRNA level of PPARγ was decreased by 61 % after HDI feeding (Table 4).

Plasma lipids and fatty acids

The triacylglycerol level in plasma and in triacylglycerol-rich lipoproteins increased by 602 and 146 %, respectively, after HDI feeding (Table 7). HDI feeding also increased the plasma level of NEFA by 121 % (Table 7).

The effects of HDI feeding on the fatty acid composition in plasma were quite different from what was seen in liver (Table 3). In general, disparate effects were seen in the changes of 16 : 0 and 18 : 1*n*-9 levels, whereas the changes in the *n*-6 and the shorter *n*-3 PUFA were similar in plasma and hepatic phospholipids after HDI feeding (Table 3). HDI feeding increased the levels of 16 : 0, 18 : 1*n*-9, 18 : 2*n*-6, 18 : 3*n*-6, 20 : 3*n*-6, 18 : 3*n*-3, 20 : 5*n*-3 and 22 : 5*n*-3 and reduced the levels of 20 : 4*n*-6 and 22 : 6*n*-3 (Table 3).

Secretion and clearance of lipids

HDI feeding decreased the mRNA levels of both AADA and apo B (Table 4). The mRNA level of hormone-sensitive lipase in epididymal WAT, and that of VLDL receptor and LPL in epididymal WAT and skeletal muscle were not changed (Table 8). HDI feeding increased the mRNA level of LDL receptor in epididymal WAT and skeletal muscle by 86 and 37 %, respectively (Table 8). The mRNA level of FAT/CD36 was increased by 60 % in epididymal WAT (Table 8), but was not changed in liver (Table 4) or in skeletal muscle (Table 8). The gene expressions of VLDL receptor and LPL in liver were down-regulated by 74 and 93 %, respectively, in rats fed HDI (Table 4).

Soya protein experiment

When obese Zucker rats were fed intact soya protein, a distinct decrease was seen in the size and number of fat droplets in liver as compared with casein-fed rats (Fig. 1). Soya protein feeding had no effect on the gene expressions of apo B and AADA, but down-regulated the mRNA levels of VLDL receptor and LPL in liver by almost 70 % (Table 9).

Discussion

When obese Zucker rats were fed a diet containing casein with a high amount of an isoflavone-enriched soya concentrate added (HDI), fewer and smaller fat droplets in liver resulted when compared with control rats fed casein. Quantitative measurements showed that the level of triacylglycerol in the liver was reduced accordingly. The reduced plasma levels of transaminases further supported that HDI feeding reduced fatty liver in this animal model.

The reduced fatty liver by HDI could be caused by increased β-oxidation, decreased lipid biosynthesis, increased secretion of VLDL and/or decreased clearance from plasma. The increased β-oxidation after HDI feeding reduced the availability of fatty acids for biosynthesis and secretion of triacylglycerol as VLDL. However, the increased lipogenesis and glycerolipid biosynthesis suggested that increased amounts of triacylglycerols and phospholipids were available for VLDL secretion. The decreased mRNA levels of AADA, a lipase involved in the mobilisation of triacylglycerol from hepatocytes to the VLDL particle (Trickett *et al.* 2001), and of apo B, provided strong indices of a reduced secretion of VLDL from the liver of HDI-fed rats. Thus, these data argue against an increased hepatic secretion of VLDL particles as the cause for the reduced fatty liver in HDI-fed rats.

The markedly down-regulated gene expression of the VLDL receptor and of LPL in liver by HDI may explain both the reduced fatty liver and the increased contents of triacylglycerol and NEFA in plasma. Normally, VLDL receptor and LPL are mainly expressed in adipose tissue and muscle, and only at very low levels in liver (Oka *et al.* 1994). As

Table 3. Selected fatty acids (shown as g/100 g fatty acids) in liver and plasma of obese Zucker rats fed a diet containing casein or a high dose of soya protein concentrate enriched with isoflavones (HDI)† (Mean values and standard deviations for six rats per group)

	16 : 0		18 : 0		16 : 1*n*-7		18 : 1*n*-9		18 : 2*n*-6		18 : 3*n*-6		20 : 3*n*-6		20 : 4*n*-6		18 : 3*n*-3		20 : 5*n*-3		22 : 5*n*-3		22 : 6*n*-3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Phospholipids																								
Casein	17·5	0·8	23·6	0·9	1·2	0·2	4·1	0·5	7·5	0·4	0·16	0·04	0·87	0·11	29·8	1·1	0·05	0·01	0·16	0·04	1·35	0·23	8·14	0·46
HDI	14·1*	1·1	26·9*	1·8	1·2	0·3	3·5	0·4	10·0*	1·8	0·31*	0·07	2·12*	0·56	27·1*	2·0	0·08*	0·02	0·53*	0·17	1·10	0·11	7·86	1·07
Triacylglycerol																								
Casein	39·5	1·8	2·5	0·1	8·6	0·9	31·5	2·4	9·2	1·0	0·39	0·06	0·06	0·01	0·28	0·08	0·37	0·04	0·04	0·01	0·09	0·03	0·09	0·03
HDI	33·8*	1·4	2·8	0·3	8·6	0·6	33·9	2·1	10·9	2·2	0·43	0·06	0·36*	0·15	0·79*	0·25	0·81*	0·24	0·13*	0·04	0·32*	0·12	0·29*	0·13
1,2-Diacylglycerol																								
Casein	39·4	2·3	3·7	1·4	5·7	1·0	26·4	2·6	10·4	1·3	0·56	0·11	0·25	0·04	3·7	1·4	0·40	0·10	0·09	0·03	1·08	0·17	2·68	0·67
HDI	25·6*	6·5	15·5*	5·9	4·6	1·4	18·3*	7·1	10·6	1·1	0·46	0·13	1·25*	0·52	12·3*	6·0	0·42	0·17	0·27*	0·10	0·90	0·21	3·87	1·98
NEFA																								
Casein	51·3	3·3	15·6	5·7	4·6	1·0	17·3	4·2	5·6	0·9	0·35	0·17	ND		2·0	0·8	ND		0·22	0·24	ND		ND	
HDI	49·0	2·9	20·7	3·8	4·3	1·0	12·6	3·6	6·6	2·5	0·41	0·26	ND		3·6*	0·7	ND		0·67	0·64	ND		ND	
Plasma																								
Casein	17·5	1·0	12·6	0·7	1·1	0·9	6·4	1·2	11·9	1·2	0·40	0·10	0·47	0·07	41·6	1·9	0·22	0·05	0·24	0·06	0·52	0·08	2·85	0·24
HDI	24·0*	2·7	11·0	2·7	3·2	2·5	19·5*	2·7	16·0*	1·9	0·51*	0·05	1·05*	0·21	13·8*	3·4	0·98*	0·21	0·57*	0·08	0·77*	0·22	1·94*	0·42

ND, not detected.
Mean values in a column for phospholipids, triacylglycerol, 1,2-diacylglycerol, NEFA or plasma were significantly different from those of the control (casein) group: *$P<0·05$.
† For details of diets and procedures, see Table 1 and p. 250.

Table 4. Hepatic mRNA levels in obese Zucker rats fed a diet containing casein or a high dose of soya protein concentrate enriched with isoflavones (HDI), presented relative to 18S rRNA and normalised to controls†

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

	Casein		HDI	
	Mean	SD	Mean	SD
AADA	1·00	0·08	0·75*	0·15
Apo B	1·00	0·12	0·71*	0·26
CPT-Ia	1·00	0·37	1·01	0·25
CPT-II	1·00	0·31	1·12	0·13
Δ5 desaturase	1·00	0·21	0·65*	0·21
Δ6 desaturase	1·00	0·19	0·96	0·28
Elongase	1·00	0·11	1·24*	0·13
FAT/CD36	1·00	0·27	1·11	0·25
L-FABP	1·00	0·24	1·11	0·25
LPL	1·00	0·39	0·07*	0·01
PPARα	1·00	0·14	0·53*	0·19
PPARδ	1·00	0·20	0·64*	0·26
PPARγ	1·00	0·40	0·39*	0·27
SCD-1	1·00	0·25	1·23	0·29
SREBP-1c	1·00	0·15	2·38*	0·72
VLDL receptor	1·00	0·37	0·26*	0·04

AADA, arylacetamide deacetylase; CPT, carnitine palmitoyltransferase; FAT/CD36, fatty acid translocase; L-FABP, liver fatty acid-binding protein; LPL, lipoprotein lipase; SCD-1, stearoyl-CoA desaturase; SREBP, sterol regulatory binding protein.

Mean values were significantly different from those of the control (casein) group: *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 250.

HDI did not affect the mRNA levels of these genes in epididymal WAT or skeletal muscle, the liver appears to be the key organ for the reduced clearance of VLDL from plasma. Furthermore, the unchanged gene expression of LPL in epididymal WAT and skeletal muscle suggested that HDI feeding did not affect the uptake of lipids to these tissues.

Table 5. Ratios of fatty acids in hepatic lipids from obese Zucker rats fed a diet containing casein or a high dose of soya protein concentrate enriched with isoflavones (HDI)†

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

	Casein		HDI	
	Mean	SD	Mean	SD
n-3/*n*-6				
Phospholipids	0·24	0·01	0·23	0·03
Triacylglycerol	0·06	0·01	0·12*	0·02
1,2-Diacylglycerol	0·27	0·02	0·20*	0·03
NEFA	0·03	0·03	0·06	0·03
20 : 4*n*-6/18 : 2*n*-6				
Phospholipids	4·00	0·34	2·82*	0·69
Triacylglycerol	0·03	0·01	0·07*	0·02
1,2-Diacylglycerol	0·35	0·12	1·17*	0·56
NEFA	0·35	0·11	0·56*	0·10
(20 : 5*n*-3 + 22 : 6*n*-3)/18 : 3*n*-3				
Phospholipids	190	39	106*	27
Triacylglycerol	0·34	0·09	0·52*	0·09
1,2-Diacylglycerol	7·0	1·5	11·7	7·2
NEFA‡	–	–	–	–

Mean values were significantly different from those of the control (casein) group: *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 250.

‡ 18 : 3*n*-3 and 22 : 6*n*-3 were not found in NEFA.

Table 6. Activities of enzymes (nmol/mg protein per min) involved in fatty acid oxidation and in biosynthesis of fatty acids and lipids in liver of obese Zucker rats fed a diet containing casein or a high dose of soya protein concentrate enriched with isoflavones (HDI)†

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

	Casein		HDI	
	Mean	SD	Mean	SD
Acyl-CoA synthetase‡	36·9	3·8	62·3*	7·9
β-Oxidation‡				
Palmitoyl-CoA	0·54	0·12	0·86*	0·05
Palmitoyl-L-carnitine	0·58	0·12	1·22*	0·30
CPT-I‡	2·9	0·3	3·2	0·3
CPT-II‡	13·6	2·4	21·2*	2·4
Acyl-CoA oxidase§	18·0	1·8	27·4*	3·1
HMG-CoA synthase‡	15·6	2·8	14·0	1·6
Acetyl-CoA carboxylase‖	9·4	1·3	17·5*	1·8
Fatty acid synthetase‖	0·43	0·05	1·16*	0·27
GPAT‡	1·2	0·2	3·3*	1·6
GPAT¶	2·1	0·5	3·2*	0·9

CPT, carnitine palmitoyltransferase; GPAT, glycerol-3-phosphate acyltransferase; HMG, 3-hydroxy-3-methylglutaryl.

Mean values were significantly different from those of the control (casein) group: *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 250.

‡ Enzyme activities were measured in mitochondria.

§ Enzyme activities were measured in peroxisomes.

‖ Enzyme activities were measured in cytosol.

¶ Enzyme activities were measured in microsomes.

HDI feeding increased the mRNA level of the LDL receptor in liver (Gudbrandsen *et al.* 2005), epididymal WAT and skeletal muscle. There is substantial evidence that the LDL receptor contributes to the clearance of not only LDL, but also of VLDL (Brown & Goldstein, 1986). However, since HDI feeding increased the triacylglycerol level both in plasma and triacylglycerol-rich lipoproteins, the LDL receptor appears to contribute little to the uptake of VLDL in these rats.

The increased plasma level of NEFA after HDI feeding was probably not due to an increased release from epididymal WAT to plasma, as the mRNA level of hormone-sensitive lipase in epididymal WAT was unchanged. The uptake of NEFA to liver, epididymal WAT and skeletal muscle via FAT/CD36 was probably not decreased, as the mRNA level of FAT/CD36 in these tissues was either unchanged or increased, and could not explain the increased plasma level of NEFA by the HDI diet.

Although the gene expression of Δ6 desaturase was not affected by HDI feeding, the reduced mRNA level of Δ5 desaturase implied that the biosynthesis of 20 : 4*n*-6 from 18 : 2*n*-6 and that of 20 : 5*n*-3 from 18 : 3*n*-3 in liver was impaired, and this was supported by the decreased product/precursor ratios of the *n*-6 and *n*-3 cascades in phospholipids. The triacylglycerols comprise most of the liver lipids in obese Zucker rats and, interestingly, HDI feeding increased the *n*-3/*n*-6, the 20 : 4*n*-6/18 : 2*n*-6 and the (20 : 5*n*-3 + 20 : 6*n*-3)/18 : 3*n*-3 ratios in liver triacylglycerols. This is an important finding since it has been shown that these ratios are decreased in patients with fatty liver (Videla *et al.* 2004). The increased product/precursor ratio and increased *n*-3/*n*-6 may favour β-oxidation over the synthesis of fatty acids and triacylglycerol, as long-chain PUFA, especially the *n*-3 PUFA, are activators of PPARα (Clarke, 2001), and thus suggest that HDI may be useful to prevent or treat fatty liver in man. Although genistein and daidzein have

Table 7. Plasma levels of triacylglycerol and NEFA, and triacylglycerol in triacylglycerol-rich lipoproteins in obese Zucker rats fed a diet containing casein or a high dose of soya protein concentrate enriched with isoflavones (HDI)†

(Mean values and standard deviations for six rats per group (in plasma) or three pooled samples of two rats each (triacylglycerol-rich lipoproteins))

	Casein		HDI	
	Mean	SD	Mean	SD
Plasma levels (mM)				
Triacylglycerol	0·83	0·14	5·83*	0·93
NEFA	0·97	0·26	2·39*	0·40
Triacylglycerol-rich lipoproteins (mol/mg protein)				
Triacylglycerol	12·8	3·4	28·3*	1·8

Mean values were significantly different from those of the control (casein) group: *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 250.

been reported to activate PPARα *in vitro* (Mezei *et al.* 2003), we found a decreased gene expression of not only PPARα, but also of PPARδ and PPARγ, after HDI feeding.

The reduced content of 20 : 4*n*-6 and the increased content of 20 : 5*n*-3 in phospholipids after HDI feeding imply that this diet may prevent the development of atherosclerosis and other inflammatory diseases, as this may favour the production of anti-inflammatory over pro-inflammatory eicosanoids.

The changes in fatty acid composition in plasma after HDI feeding was not similar to what was seen in any lipid fraction isolated from liver. Since triacylglycerol-rich lipoproteins from obese Zucker rats fed casein contain approximately 80 % triacylglycerol (OA Gudbrandsen, H Wergedahl and RK Berge, unpublished results), the triacylglycerols will contribute to a majority of the fatty acids in plasma. Certain analogous changes were seen in plasma and liver phospholipids, especially regarding the *n*-6 and *n*-3 PUFA, possibly because a sizeable portion of VLDL triacylglycerol arise from cellular fatty acids esterified as phospholipids (Wiggins & Gibbons, 1996). The markedly increased 18 : 1*n*-9 and decreased 20 : 4*n*-6 levels in plasma after HDI feeding were not seen in any liver lipids except for cholesteryl esters (Gudbrandsen *et al.* 2005), supporting findings showing that cholesteryl esters of VLDL are non-selectively transferred from the liver (Gidez *et al.* 1965).

We have previously demonstrated that dietary soya protein lowers the hepatic lipid content in obese Zucker rats (Wergedahl *et al.* 2004). In the present study we show that the mechanism of action of the soya protein is similar to that of HDI, as soya protein appears to enhance β-oxidation (Wergedahl *et al.* 2004) and probably does not increase the secretion of VLDL from liver, as the mRNA levels of AADA and apo B were not changed. In addition, soya protein feeding decreased the mRNA levels of VLDL receptor and LPL, indicating that also with intact soya protein the reduced fatty liver might be due to decreased clearance of VLDL from plasma. Since the amino acid compositions of casein and HDI diets were almost similar (Gudbrandsen *et al.* 2005), while that of soya protein was markedly different (Wergedahl *et al.* 2004), this suggests that the isoflavones and not the amino acids may be the bioactive components of the HDI diet.

To conclude, the reduced fatty liver in obese Zucker rats fed HDI or soya protein appear to be due to increased catabolism of fatty acids in liver combined with reduced clearance of VLDL from the circulation by the liver.

Table 8. mRNA levels in epididymal white adipose tissue (WAT) and skeletal muscle of obese Zucker rats fed a diet containing casein or a high dose of soya protein concentrate enriched with isoflavones (HDI), presented relative to 18S rRNA and normalised to controls†

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

	Casein		HDI	
	Mean	SD	Mean	SD
Epididymal WAT				
FAT/CD36	1·00	0·24	1·60*	0·14
HSL	1·00	0·23	0·86	0·24
LPL	1·00	0·17	1·01	0·12
LDL receptor	1·00	0·13	1·86*	0·47
VLDL receptor	1·00	0·11	0·99	0·12
Skeletal muscle				
FAT/CD36	1·00	0·10	1·18	0·27
LPL	1·00	0·19	0·94	0·16
VLDL receptor	1·00	0·10	0·90	0·09
LDL receptor	1·00	0·22	1·37*	0·25

FAT/CD36, fatty acid translocase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase.

Mean values were significantly different from those of the control (casein) group: *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 250.

Table 9. mRNA levels in liver of obese Zucker rats fed a diet containing casein or soya protein, presented relative to 18S rRNA and normalised to controls†

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

	Casein		Soya protein	
	Mean	SD	Mean	SD
AADA	1·00	0·11	0·96	0·11
Apo B	1·00	0·10	0·91	0·07
LPL	1·00	0·25	0·31*	0·08
VLDL receptor	1·00	0·43	0·32*	0·16

AADA, arylacetamide deacetylase; LPL, lipoprotein lipase.

Mean values were significantly different from those of the control (casein) group: *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 250.

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