Effect of dietary methionine on plasma and liver cholesterol concentrations in rats and expression of hepatic genes involved in cholesterol metabolism

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Methionine has been shown to increase plasma cholesterol in animals. In the present study, mechanisms were investigated by which methionine could alter cholesterol metabolism. In the first experiment, forty growing rats were fed four casein-based diets differing in methionine content (2·6, 3·5, 4·5 or 6·0 g/kg) for 14 d. In the second experiment, isolated rat hepatocytes were incubated in media supplemented with 50, 100 or 200 μmol/l methionine. Dietary methionine tended to increase plasma homocysteine concentrations in the rats ($P = 0.058$). A weak positive correlation between circulating homocysteine and plasma cholesterol was observed (R^2 0·27, $P < 0.01$). Rats fed 3·5 g/kg or more of methionine had higher concentrations of cholesterol in their plasma, in lipoprotein fractions of density (ρ; kg/l) 1·006 < ρ < 1·063 and ρ > 1·063, and in liver than rats fed 2·6 g/kg methionine. Rats fed 6 g/kg methionine had a higher hepatic expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol-7α-hydroxylase than rats fed less methionine. The phosphatidylcholine:phosphatidylethanolamine ratio in rat liver increased with rising dietary methionine concentration; the relative mRNA concentrations of phosphatidylethanolamine N-methyltransferase and cystathionine β-synthase remained unaffected. Hepatocytes incubated in media supplemented with 100 or 200 μmol/l methionine had a higher cholesterol synthesis than hepatocytes incubated in a medium supplemented with 50 μmol/l methionine; the LDL uptake in hepatocytes was independent of the methionine concentration of the medium. In conclusion, the present study suggests that dietary methionine induces hypercholesterolaemia at least in part via an enhanced hepatic cholesterol synthesis.

Methionine: Homocysteine: Cholesterol metabolism: Liver: Rat

Previous studies on the effects of dietary proteins on plasma cholesterol have shown that casein raises serum and LDL-cholesterol concentration in animals and man, whereas plant proteins such as soya protein do not (Ikeda *et al.* 1993; Kritchevsky, 1993; Sirtori *et al.* 1993; Sugano & Koba, 1993; Carroll & Kurowska, 1995). The results obtained from these studies suggest that alterations in plasma cholesterol concentration could be mediated by differences in the amino acid patterns of the dietary proteins. Methionine is one of the amino acids that could contribute to this phenomenon because it is more abundant in casein than in soya protein. Sugiyama *et al.* (1998) have shown that high concentrations of dietary methionine increase plasma phosphatidylcholine, the phosphatidylcholine:phosphatidylethanolamine ratio of liver microsomes and the cholesterol concentration in rat plasma. In support of this finding, Giroux *et al.* (1999) reported that concentrations of hepatic phosphatidylcholine and plasma cholesterol were higher in rabbits fed a diet enriched with L-lysine and L-methionine.

Both research groups proposed that hypercholesterolaemia induced by high dietary methionine concentrations might be caused by alterations in hepatic phospholipid metabolism.

Dietary methionine is mainly metabolised in the liver, where it is converted to S-adenosylmethionine (SAM). SAM functions as methyl donor for the methylation of phosphatidylethanolamine into phosphatidylcholine. It is suggested that methionine could influence the formation of phosphatidylcholine by increasing the availability of methyl groups from SAM via a phosphatidylethanolamine N-methyltransferase (PEMT)-mediated pathway. Phosphatidylcholine is the major phospholipid class of plasma lipoproteins and is required for the assembly and secretion of apo-B-containing lipoproteins from liver into the plasma (Yao & Vance, 1988). On the other hand, the phosphatidylcholine:phosphatidylethanolamine ratio in the liver seems to have a regulatory function in cholesterol metabolism because previous findings have demonstrated that methionine-induced hypercholesterolaemia is completely abolished by supplementation with eritadenine, a potent inhibitor of phosphatidylethanolamine N-methylation (Sugiyama *et al.* 1998). Additionally, it is known that canalicular membrane phosphatidylcholine synthesis in liver, in concert with the phospholipid transporter, promotes the excretion of phospholipid and cholesterol into the bile (Sehayek *et al.* 2003), a mechanism that normally contributes to a reduction in cholesterol concentration in the plasma.

By donating its labile methyl group, SAM becomes S-adenosylhomocysteine (SAH), which can be remethylated to SAM or converted into cysteine, a reaction that depends on the action of cystathionine β-synthase. SAH can be converted to homocysteine. Recent findings indicate that homocysteine has a hypercholesterolaemic action by stimulating cholesterol biosynthesis via activation of the transcription factors sterol regulatory element-binding protein-2, cAMP response element-binding protein and nuclear factor Y in the liver (Woo *et al.* 2005). It is also possible therefore that the hypercholesterolaemic action of methionine is caused by the homocysteine-induced stimulation of cholesterol biosynthesis. The expression of the LDL receptor gene, another sterol regulatory element-binding protein-2-dependent gene, has not yet been investigated in this respect. However, the molecular basis of the effect of methionine on the cholesterol metabolism is not yet fully understood.

The present study was therefore undertaken to find out more about the effects of dietary methionine on cholesterol metabolism. Growing rats were used as the model. To explore possible dose-dependent effects of dietary methionine, we used four diets with methionine concentrations of 2·6, 3·5, 4·5 and 6·0 g/kg. The diet with the methionine concentration of 4·5 g/kg matched the recommended intake for growing rats (AIN-93G; Reeves *et al.* 1993). Diets with 2·6 g/kg and 3·5 g/kg methionine represented low-methionine diets. The diet containing 6·0 g/kg methionine was representative of a moderate methionine excess. All the diets met the minimum requirement for methionine and sulphur-containing amino acids to prevent growth retardation in growing rats. A possible effect of dietary methionine on cholesterol excretion was investigated by determining the gene expression for cholesterol-7α-hydroxylase (CYP7A1) in the liver, the key enzyme in the synthesis of bile acids from cholesterol, and by measuring bile acids in the faeces. We also tested whether dietary methionine could alter the synthesis of phosphatidylcholine and phosphatidylethanolamine and the expression of PEMT, one of the main enzymes involved in phosphatidylcholine synthesis. We suggested that an alteration of the phosphatidylcholine:phosphatidylethanolamine ratio might alter membrane fluidity and, as a consequence, fatty acid desaturation. We therefore determined the gene expression of Δ6-desaturase in liver and the ratio of arachidonic to linoleic acid, an indicator of Δ6-desaturase activity in liver phospholipids.

Cholesterol biosynthesis in the liver and the uptake of LDL from the plasma into the cells via the LDL receptor are other important processes involved in the regulation of plasma cholesterol concentration. A determination of the relative mRNA concentration in the liver of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol biosynthesis, and the relative mRNA concentration of the LDL receptor in rat liver should provide further information about the effects of methionine on cholesterol metabolism *in vivo*. To ascertain the possible effects of methionine on the uptake of LDL by the LDL receptor and on cholesterol biosynthesis, we additionally performed a study with primary rat hepatocytes cultivated in media with different concentrations of methionine. Primary rat hepatocytes were used as *in vitro* model to obtain further data about possible mechanisms by which methionine could alter cholesterol concentrations in rats. The concentrations of homocysteine in the

plasma and liver of rats in response to diets with a different methionine concentration were measured in order to elucidate a potential link between dietary methionine, plasma homocysteine and plasma cholesterol.

Materials and methods

Feeding experiment

Forty 4-week-old male Sprague-Dawley rats were obtained from Charles River (Sulzfeld, Germany). They were housed individually in Macrolon cages in a temperature- ($23 \pm 1°$C) and humidity-controlled (50–60%) room with a 12 h light/dark cycle (10.00 hours to 22.00 hours). The rats were acclimatised to the facility for 7 d and given free access to a stock diet (Altromin, Lage, Germany). At 5 weeks of age, the rats were randomly assigned to one of four groups of ten rats each with similar initial mean body weights (group 1, 123 (SEM 2·2) g; group 2, 122 (SEM 2·9) g; group 3, 122 (SEM 3·3) g; group 4, 123 (SEM 3·1) g). The experiment followed established guidelines for the care and handling of laboratory animals approved by the council of Saxony-Anhalt.

A basal semisynthetic diet was used. The composition of the experimental diets is shown in Table 1. Diets were prepared by mixing the dry components with fat and water, followed by freeze-drying. The residual water content of the diets was below 5 g per 100 g diet. The composition of the vitamin and mineral mixture followed recommendations from the National Research Council (1995). Casein was obtained from Meggle (Wasserburg, Germany), L-methionine from Sigma-Aldrich (Deisenhofen, Germany) and the other amino acids from Lohmann Animal Health (Cuxhaven, Germany). The amount of total sulphur amino acids (methionine and cysteine) in the diets was maintained at a constant level on a molar basis, equivalent to an amount of methionine of 8 g/kg diet. No supplemental methionine was added to the diet containing 2·6 g/kg methionine. The other diets were

Table 1. Composition of experimental diets differing in methionine

| | Experimental diets | | | |
| | Dietary methionine concentration (g/kg) | | | |
Ingredient	2·6	3·5	4·5	6·0
Casein	100	100	100	100
Free amino acid mixture*	87·1	87·1	87·1	87·1
Maize starch	490·8	490·7	490·5	490·2
Sucrose	215	215	215	215
Maize oil	50	50	50	50
Cellulose	20	20	20	20
Vitamin and mineral mixture†	29	29	29	29
Choline chloride	4	4	4	4
L-methionine	0	0·9	1·9	3·4
Cysteine	4·1	3·3	2·5	1·3

*The following amino acids were supplemented (g/kg diet): lysine*HCl, 8·27; threonine, 3·38; arginine*HCl, 4·37; histidine, 2·51; isoleucine, 4·51; leucine, 7·75; phenylalanine, 4·25; valine, 5·51; aspartic acid, 5·75; glutamic acid, 17·5; glycine, 1·49; proline, 9·38; serine, 4·25; tyrosine, 4·63; alanine, 2·51; tryptophan, 1·05.
† The vitamin and mineral mixture was composed according to recommendations from the National Research Council (1995).
For details of diets and procedures, see this page.

supplemented with L-methionine to bring the total methionine content to 3·5, 4·5 and 6·0 g/kg (Table 1).

All the diets were supplemented with cysteine to contain 4·5, 3·7, 2·9 and 1·7 g/kg cysteine (Table 1). The other amino acids were added in the form of a free amino acid mixture and did not differ between the four experimental diets (Table 1). The total dietary concentrations of the other amino acids were (g/kg diet): lysine, 13·3; threonine, 7·3; arginine, 6·8; histidine, 5·7; isoleucine, 8·9; leucine, 16·6; phenylalanine, 9·1; valine, 11·5; aspartic acid, 12·4; glutamic acid, 38·6; glycine, 3·2; proline, 19·5; serine, 9·6; tyrosine, 9·0; alanine, 5·3. The concentration of tryptophan was not determined for technical reasons. Based on literature values for casein and the amount supplemented, the tryptophan concentration was estimated to be 2·2 g/kg.

The rats were fed once daily at 09.00 hours. Diets were supplied *ad libitum*. The daily feed intake was recorded by weighing the amount of diet not eaten by the rats. Water was available from nipple drinkers.

Sample collection and preparation

After a feeding period of 14 d, the non-fasted rats were killed by decapitation under light anaesthesia with diethyl ether (09.00 hours to 11.00 hours). Rats were not fasted before killing because food deprivation leads to a significant downregulation of the genes encoding enzymes involved in lipid metabolism (Shimano *et al.* 1999), which were to be measured in the present study. Plasma was separated from heparinised whole blood by centrifugation at $1880\,g$ for 10 min at 4°C. Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at $900\,000\,g$ at 4°C for 1·5 h. The lipoprotein fractions were collected on the basis of their densities (ρ; kg/l) as described previously for rats (Giudetti *et al.* 2003): $\rho < 1·006$ kg/l, $1·006$ kg/l $< \rho < 1·063$ kg/l and $\rho > 1·063$ kg/l. Plasma densities were adjusted with NaCl and KBr. The lipoprotein fractions used for cholesterol analysis were removed by suction. Livers were excised, weighed and immediately snap-frozen in liquid N. Aliquots of liver for RNA isolation were stored at -80°C; other samples were stored at -20°C. During the second week of the experimental period, faeces were collected, dried, weighed and stored at -20°C until analysis.

Lipid analyses

Lipids from liver and cultivated cells were extracted with a mixture of n-hexane and isopropanol (3:2, v/v; Hara & Radin, 1978). The concentrations of lipids in liver were determined using an enzymatic reagent kit after drying an aliquot of the lipid extracts and dissolving the lipids with Triton X-100 (De Hoff *et al.* 1978). Concentrations of cholesterol in the liver, plasma and lipoprotein fractions were determined using an enzymatic reagent kit (Cat.-No. 1·14 830; VWR International, Darmstadt, Germany).

The phosphatidylcholine and phosphatidylethanolamine fractions of the extracts were separated by normal-phase HPLC equipped with a fraction collector using a Kromasil column (12·5 cm × 0·46 cm, Si 5 μm, 100 nm; Chromatographie Service, Langerwehe, Germany) and an elution system consisting of n-hexane–isopropanol (80:20, v/v) and methanol (Christie, 1985). After evaporation of the elution solvents using a centrifugal evaporator, phosphatidylcholine and phosphatidylethanolamine were methylated with trimethylsulphonium hydroxide (Butte, 1983). Fatty acid methyl esters were separated by GC (Eder & Brandsch, 2002). The ratio of arachidonic acid (20: 4n-6) to linoleic acid (18: 2n-6) was calculated on a molar basis. The concentrations of phosphatidylcholine and phosphatidylethanolamine in the lipid extracts were determined by quantitative high-performance thin-layer chromatography on HPTLC plates Si60 (Cat.-No. 1·05 633; VWR International). Samples and standards were applied with a Linomat 5, and the chromatography plates were developed in a horizontal chamber (both from Camag, Muttenz, Switzerland) with a mixture of chloroform–methanol–concentrated ammonia (26:10:1·6, v/v/v). Lipids were visualised by dipping in 10 % aqueous ammonium sulphate and stained at 200°C. Spots were evaluated densitometrically using video equipment and the corresponding software from Syngene (Cambridge, UK). The ratio of phosphatidylcholine:phosphatidylethanolamine was calculated on a molar basis.

The content of total bile acids in the faeces was determined enzymatically (DiaSys Diagnostic Systems, Holzheim, Germany) by a modified method of Marlett & Fischer (2002) using taurocholic acid as a standard. For that purpose, the bile acids were extracted from freeze-dried faeces with ethanol and NaOH as recently described (Dongowski *et al.* 2002).

Analyses of amino acids, homocysteine, S-adenosylmethionine and S-adenosylhomocysteine

The amino acid concentrations of the diets were determined with an amino acid analyser (LC3000; Eppendorf, Hamburg, Germany) after the diets had been digested with HCl (Bassler & Buchholz, 1993). The concentrations of free amino acids in the plasma and in cell culture media were measured as isoindole derivatives by HPLC (1100 series; Agilent Technologies, Waldbronn, Germany) according to Schuster (1988) after pre-column derivatisation (Teerlink *et al.* 1994). Amino acid isoindoles were detected at an excitation wavelength of 337 nm and an emission wavelength of 454 nm.

Total homocysteine and total cysteine were also determined by HPLC (Vester & Rasmussen, 1991). To measure the hepatic homocysteine concentrations, aliquots of liver were thawed and homogenised in ice-cold PBS; the homocysteine concentrations were determined by HPLC according to the method of Vester & Rasmussen (1991). The concentrations of SAM and SAH in the liver were measured by HPLC according to Sugiyama *et al.* (1995). Samples of frozen liver were thawed and homogenised in 0·5 mol/l ice-cold perchloric acid, and the homogenates were centrifuged at $16\,000\,g$ for 20 min at 4°C. The resultant supernatants were applied to a HPLC column (Hypersil ODS 250×4 mm^2, 5 μm; Agilent Technologies). The mobile phase was a 100 mmol/l potassium dihydrogen phosphate solution containing 10 mmol/l sodium heptane sulphonate and 3 % (v/v) methanol. The flow rate was 1·5 ml/min, and the elution was monitored at 254 nm.

Gene expression analyses

For analysis of gene expression, total RNA was extracted from frozen liver samples using Trizol reagent (Invitrogen, Karlsruhe, Germany). RNA was quantified by A_{260} and its integrity verified by agarose gel electrophoresis using ethidium bromide for visualisation. Total RNA and oligo dT primer (Amersham Pharmacia, Freiburg, Germany) were used for cDNA synthesis (Omniscript RT Kit from Qiagen, Hilden, Germany; Mastercycler Personal from Eppendorf). The concentration of cDNA was analysed by real-time detection PCR using Sybr green I (Rotorgene 2000; Corbett Research, Mortlake, Australia). The amplification efficiency and the take-off point calculated by Rotorgene software 4·6 were used for comparative quantification. Different runs were made comparable by purified PCR products, with the extraction of cut ethidium bromide-stained bands following 2 % agarose gel electrophoresis by MiniElute Gel Extraction Kit (Qiagen) used as a standard. The expression signal of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase served as internal control for normalisation. PCR was carried out using 100 μl Rotorgene PCR tubes with a final volume of 15 μl reaction mixture containing 500 μmol/l dNTP (Roth, Karlsruhe, Germany), 3·5 mmol/l $MgCl_2$, 5 U Taq DNA polymerase and 1·5 μl 10 × buffer (all from Promega, Mannheim, Germany), 0·38 μl 10 x Sybr Green I (Sigma-Aldrich), 2 μl first-strand cDNA or standard, and 2 μl primer mix. The primer oligonucleotides were selected using Primerselect software (DNA-Star Inc., Madison, WI, USA) and Abi-Prism Primerpairs software (Applied Biosystems, Weiterstadt, Germany) from database sequences (Roth).

The sequences, product length and concentrations in the stock solutions were:

1. glyceraldehyde-3-phosphate dehydrogenase (EC 1·2·1·12; EMBL ID: RNAGAPDHR), up: 5′-GCA TGG CCT TCC GTG TTC C-3′, low: 5′-GGG TGG TCC AGG GTT TCT TAC TC-3′; 337 bp, 1·5 pmol/μl;
2. PEMT (EC 2·1·1·17; NCBI GenBank: NM 013003), up: 5′-GCC CAG CTC CCA TTT CCT TC-3′, low: 5′-CAT CTT GGG CTG GCT CAT CAT-3′; 286 bp, 10 pmol/μl;
3. CYP7A1 (EC 1·14·13·17; NCBI GenBank: J05509), up: 5′-CAA GAC GCA CCT CGC TAT CC -3′, low: 5′-CCG GCA GGT CAT TCA GTT G-3′; 206 bp, 10 pmol/μl;
4. cystathionine β-synthase (EC 4·2·1·22; NCBI GenBank: NM 012522), up: 5′-CAG TGC GAC GGG AAG GTG GAC A -3′, low: 5′-ATG CGG GCG AAG GCG AAG GA -3′; 284 bp, 5 pmol/μl;
5. HMG-CoA reductase (EC 1·1·1·34), up: 5′-CAG GGA ACA TGC ACC AAG AAG G-3′, low: 5′-ATA CGG CAC GGA AAG AAC CAT AGT-3′; 237 bp, 10 pmol/μl;
6. LDL receptor (NCBI GenBank: X13722), up: 5′-AGT GGC CGC CTC TAT TGG GTT GAT-3′, low: 5′-TCC GGG GAC ATG AGG TTT TTA GC-3′; 236 bp, 2·5 pmol/μl;
7. Δ6-desaturase (EC 1·14·99; EMBL ID: AB621980), up: 5′-CTT TCT CCT CCT GTC CCA CAT-3′, low: 5′-CAT TGC CGA AGT ACG AGA GGA-3′; 71 bp, 7·5 pmol/μl.

The DNA of glyceraldehyde-3-phosphate dehydrogenase, PEMT, CYP7A1, cystathionine β-synthase, HMG-CoA reductase, LDL receptor and Δ6-desaturase was amplified in cycles of 20 s denaturation at 95°C, 30 s annealing at 60°C and 40 s elongation at 72°C. Fluorescence was measured at 72°C. A final melting curve guaranteed the authenticity of the target product.

Cell culture study

Hepatocytes were taken from six male Sprague-Dawley rats (6 weeks old, 150–180 g), which were obtained from Charles River and fed a stock diet (Altromin). Hepatocytes were prepared by the collagenase perfusion technique (Seglen, 1976). The viability of the isolated cells, checked by trypan blue exclusion assay, was more than 90 %.

Cells were seeded into collagen-coated 24-well cell culture plates (Greiner Bio-one, Frickenhausen, Germany) to a density of 0.5×10^6 cells per well and millilitre in RPMI 1640 medium (with a basal concentration of 100 μM-methionine) supplemented with 10 % fetal bovine serum, 50 mg/l gentamicin (all from Invitrogen) and insulin (0·6 mg/l) from porcine pancreas (Sigma-Aldrich). Cells were cultivated at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2. After 2–4 h, the medium was removed and replaced by 400 μl methionine-free RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 50, 100 or 200 μmol/l methionine, 10 % fetal bovine serum, 50 mg/l gentamicin and 0·6 mg/l insulin. The final methionine concentrations in the media that resulted from supplementation with methionine and fetal bovine serum were 53, 103 and 203 μmol/l, respectively. The medium containing 53 μmol/l methionine was used as control and corresponded to the plasma concentrations of methionine that were measured in the rat groups fed the low-methionine diets. After a further 24 h, the cells were used to measure the phospholipid composition of the cells, the LDL uptake, the rate of cholesterol synthesis and the concentrations of homocysteine and methionine in the medium.

Measurement of LDL uptake

LDL ($1.006 < \rho < 1.063$; kg/l) was isolated by ultracentrifugation of EDTA plasma from normal lipidaemic human donors and labelled with the fluorochrome 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) according to Zouhair & Gurachek (1993) with modifications: 0·6 ml LDL dialysed against PBS containing 0·1 mg/ml EDTA (mean protein concentration 800 μg/ml) was mixed with a 100 mmol/l solution of ascorbic acid (1 μl) to prevent oxidation and 2 μl DiI in dimethyl sulphoxide (33·6 mg/ml). The mixture was incubated for 6 h at 37°C in the dark, under N gas and with gentle agitation. The DiI-labelled LDL was reisolated after sterile filtration (0·2 μm) and density adjustment as an upper layer by ultracentrifugation and dialysed against PBS under N for 12 h.

For uptake studies, cells were washed with RPMI 1640 medium without supplements and incubated with 200 μl RPMI 1640 containing 10 μg DiI-LDL protein for 2 h at 37°C. After incubation, cells were washed twice with cold PBS, and lipids were extracted with 300 μl isopropanol. The extracts were centrifuged at 10 000 g for 5 min, and the fluorescence was measured in the supernatant with the fluorescence detector of the 1100-HPLC (Agilent Technologies; excitation wavelength 520 nm, emission wavelength 580 nm). All incubations were replicated four times per animal. To determine

the unspecific binding, cells were incubated with LDL in the presence of heparin (80 000 U/l), which inhibits the LDL receptor-mediated uptake of LDL (Goldstein *et al.* 1976). The specific uptake was calculated by subtracting the unspecific binding from the total uptake.

Cellular protein concentration was measured after isopropanol extraction by solubilising the protein in 0·2 mol/l NaOH followed by the bicinchoninic acid assay. Uptake was expressed as ng LDL-protein per mg cell protein per 2 h.

Analysis of in vitro *cholesterol synthesis*

Newly synthesised cholesterol was measured by the addition of 0·05 μCi [1,2-^{14}C] acetate (specific activity 108 mCi/mmol; Hartmann Analytic, Braunschweig, Germany) to the culture media according to Mehran *et al.* (1995) and Lovati *et al.* (2000). Cells were incubated for 4 h at 37°C. All incubations were replicated six times per animal. After incubation, the cells were washed twice with cold PBS and the culture plates stored at −20°C pending analysis.

The lipids were extracted twice with a mixture of hexane and isopropanol (3:2, v/v; Liscum & Faust, 1987). After removing the solvents in a vacuum centrifugal evaporator (Jouaie, Saint-Herblain, France) the lipids were dissolved in 50 μl chloroform, 3 μl of which were applied to 10 × 20 cm^2 TLC sheets (Si 60 aluminium sheets, Cat.-No. 1·05 553·0001; VWR International) using a TLC spotter PS01 (Desaga, Heidelberg, Germany). Plates were developed with a mixture of hexane, diethyl ether and acetic acid (80:20:3, v/v/v; Levy *et al.* 1992). Lipid-bound radioactivity was detected and quantified by autoradiography (Fuji imager system, Tina 2 software; Raytest, Straubenhart, Germany) in relation to [4-^{14}C] cholesterol standards (Biotrend Chemikalien GmbH, Cologne, Germany). Cellular protein content was measured after the lipid extraction by solubilisation of protein in 0·2 mol/l NaOH solution using the bicinchoninic acid assay.

Statistical analysis

Data were analysed by ANOVA using Minitab Statistical Software (Minitab, State College, PA, USA). Differences between means were tested using Fisher's multiple range test when the F value was significant at $P < 0.05$. Data were considered significantly different at $P < 0.05$. A linear regression analysis was performed between plasma homocysteine and plasma cholesterol concentrations.

Results

Food intake and growth development of the rats

Mean food intake (group 1: 19·7 (SEM 0·5) g/d; group 2: 19·1 (SEM 0·4) g/d; group 3: 18·6 (SEM 0·5) g/d; group 4: 18·9 (SEM 0·6) g/d; *n* 10) and body weight gain (group 1: 120 (SEM 4) g; group 2: 120 (SEM 3) g; group 3: 115 (SEM 5) g; group 4: 122 (SEM 4) g; *n* 10) did not differ between the four groups of rats.

Concentrations of amino acids in rat plasma and liver

The methionine concentration in the plasma increased as the dietary methionine concentration rose (Table 2). The homocysteine concentrations in the plasma of rats fed 4·5 or 6·0 g/kg methionine tended to be higher ($P = 0.058$) than the homocysteine concentrations in the plasma of rats fed 2·6 or 3·5 g/kg methionine (Table 2). Rats fed a diet with 3·5 g/kg methionine or more had lower concentrations of lysine ($P < 0.05$) and higher concentrations of histidine ($P < 0.05$) in their plasma than rats fed the diet with 2·6 g/kg methionine (lysine, group 1: 725 (SEM 67) μmol/l; group 2: 489 (SEM 15) μmol/l; group 3: 563 (SEM 27) μmol/l; group 4: 545 (SEM 30) μmol/l; histidine, group 1: 97·2 (SEM 6·8) μmol/l; group 2: 75·2 (SEM 4·1) μmol/l; group 3: 79·0 (SEM 5·0) μmol/l; group 4: 78·0 (SEM 3·3) μmol/l; *n* 10). The plasma concentrations of serine, taurine, cystine, ethanolamine, phenylalanine, tryptophan, leucine, isoleucine, valine, tyrosine, alanine, threonine, arginine, glutamine, glycine, glutamic acid and asparagine were no different between the four groups of rats (data not shown). Concentrations of homocysteine and SAM in the liver did not differ between the four groups of rats (Table 2). Rats fed the high-methionine diet with 6·0 g/kg methionine had higher concentrations of SAH in their livers than groups fed 2·6 or 3·5 g/kg dietary methionine (Table 2). The ratio of SAH to SAM in liver did not differ between the four groups of rats (Table 2).

Table 2. Concentrations of methionine and homocysteine in plasma and concentrations of homocysteine, SAM and SAH in liver of growing rats fed diets with different concentrations of methionine

(Mean values with their standard errors for ten rats per group)

Dietary methionine (g/kg)	2·6 Mean	2·6 SEM	3·5 Mean	3·5 SEM	4·5 Mean	4·5 SEM	6·0 Mean	6·0 SEM
Plasma								
Methionine (μmol/l)	50·1	3·2[b]	54·3	4·0[b]	76·1	7·1[a]	81·9	8·5[a]
Homocysteine (μmol/l)	5·98	·47	6·02	0·51	8·03	0·62	8·62	1·37
Liver								
Homocysteine (nmol/g)	5·0	0·5	4·3	0·4	5·3	0·6	4·4	0·4
SAM (nmol/g)	123	12	125	9	142	9	150	9
SAH (nmol/g)	24·2	1·6[b]	24·2	2·2[b]	28·8	2·4[a,b]	32·6	1·7[a]
SAH:SAM ratio (nmol/nmol)	0·21	0·02	0·20	0·02	0·21	0·02	0·22	0·02

SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
For details of diets and procedures, see p. 880.

Lipid concentrations in plasma, lipoproteins and liver of rats

Concentrations of cholesterol in the plasma, liver and lipoprotein fractions with densities (kg/l) of $1{\cdot}006 < \rho < 1{\cdot}063$ and $\rho > 1{\cdot}063$ were higher in rats fed the diets with 3·5 g/kg methionine or more than in rats fed the low-methionine diet containing 2·6 g/kg methionine (Table 3). The concentration of cholesterol in the lipoprotein fraction with a density (kg/l) of $\rho < 1{\cdot}006$ was not significantly different between the groups. The phosphatidylcholine:phosphatidylethanolamine ratio in the liver increased as the dietary methionine concentration rose (Table 3). The ratio of 20 : 4*n*-6:18 : 2*n*-6 fatty acid in phosphatidylcholine was higher in the rats fed diets containing 4·5 or 6·0 g/kg methionine than in rats fed diets containing 2·6 g/kg methionine (Table 3). The ratio of 20 : 4*n*-6:18 : 2*n*-6 in phosphatidylethanolamine did not differ between the four groups of rats.

Correlation between plasma homocysteine and plasma cholesterol concentrations

Regression analysis showed a weak positive correlation between the concentrations of plasma homocysteine and plasma cholesterol ($P < 0{\cdot}01$; Fig. 1).

Faecal excretion of bile acids

The excretion of bile acids via the faeces was no different between the four groups of rats (group 1: 83·1 (SEM 7·4) μmol/week; group 2: 80·2 (SEM 7·0) μmol/week; group 3: 69·7 (SEM 4·8) μmol/week; group 4: 86·3 (SEM 7·1) μmol/week; *n* 10).

Gene expression of hepatic enzymes and LDL receptor in rats

The relative mRNA concentration of HMG-CoA reductase in the liver increased with rising dietary methionine concentration (Fig. 2(a)). The relative gene expression of PEMT in the liver did not differ between the four groups of rats (group 1: 0·91 (SEM 0·26); group 2: 1·24 (SEM 0·29); group 3: 1·00 (SEM 0·21); group 4: 0·70 (SEM 0·11); *n* 10).

The relative mRNA concentration of CYP7A1 in the liver was higher in rats fed diets with 6·0 g/kg methionine than in rats fed diets with 2·6 or 3·5 g/kg methionine (Fig. 2(b)). Rats fed the diet with 4·5 g/kg methionine did not differ from rats fed the diet with 2·6, 3·5 or 6·0 g/kg methionine diet in terms of their relative mRNA concentration of CYP7A1 (Fig. 2(b)).

Relative gene expressions of cystathione β-synthase (group 1: 0·93 (SEM 0·10); group 2: 1·28 (SEM 0·26); group 3: 1·00 (SEM 0·21); group 4: 1·05 (SEM 0·21); *n* 10), Δ6-desaturase (group 1: 1·24 (SEM 0·30); group 2: 1·13 (SEM 0·18); group 3: 1·00 (SEM 0·17); group 4: 1·84 (SEM 0·41); *n* 10) and the LDL receptor (group 1: 1·00 (SEM 0·30); group 2: 1·28 (SEM 0·46); group 3: 1·00 (SEM 0·32); group 4: 1·21 (SEM 0·46); *n* 10) in the liver did not differ between the four groups of rats.

Phosphatidylcholine:phosphatidylethanolamine ratio, cholesterol synthesis and LDL uptake in rat hepatocytes

The phosphatidylcholine:phosphatidylethanolamine ratio and LDL uptake did not differ between cells incubated in media supplemented with 50 (control medium), 100 or 200 μmol/l methionine (Table 4). However, cells incubated in media supplemented with 100 or 200 μmol/l methionine had a higher rate of cholesterol synthesis than cells incubated in the control medium supplemented with 50 μmol/l methionine. After incubating the cells for 24 h, the concentrations of homocysteine in the cell culture media increased significantly with increasing amounts of supplemented methionine (Table 4).

Discussion

The present study shows that methionine added to a diet containing the minimum level of methionine required for rat

Table 3. Concentration of cholesterol in the plasma, lipoprotein fractions and liver, phosphatidylcholine:phosphatidylethanolamine ratio in the liver and ratio of 20 : 4*n*-6:18 : 2*n*-6 in the liver phospholipids of growing rats fed diets with different concentrations of methionine

(Mean values with their standard errors for ten rats per group)

Dietary methionine (g/kg)	2·6		3·5		4·5		6·0	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total cholesterol								
Plasma (mmol/l)	2·13	0·11[b]	2·51	0·09[a]	2·72	0·11[a]	2·53	0·11[a]
Lipoprotein fractions (mmol/l)								
$\rho < 1{\cdot}006$	0·32	0·05	0·36	0·03	0·40	0·04	0·38	0·02
$1{\cdot}006 < \rho < 1{\cdot}063$	0·61	0·05[b]	0·76	0·05[a]	0·82	0·05[a]	0·76	0·04[a]
$\rho > 1{\cdot}063$	1·17	0·06[b]	1·36	0·07[a,b]	1·47	0·07[a]	1·49	0·11[a]
Liver (μmol/g)	5·62	0·61[a]	8·64	1·37[a,b]	9·92	1·32[b]	9·41	1·42[b]
Phospholipids								
Liver phosphatidylcholine: phosphatidylethanolamine ratio (mol/mol)	2·62	0·04[b]	2·91	0·23[a,b]	3·50	0·31[a,b]	3·75	0·37[a]
Fatty acids								
Liver phosphatidylcholine: 20 : 4*n*-6:18 : 2*n*-6 ratio (mol/mol)	3·08	0·26[b]	3·97	0·73[a,b]	4·35	0·17[a]	4·53	0·20[a]
Liver phosphatidylethanolamine: 20 : 4*n*-6:18 : 2*n*-6 ratio (mol/mol)	2·72	0·07	3·09	0·11	2·95	0·07	3·03	0·11

ρ, density (kg/l).
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0{\cdot}05$).
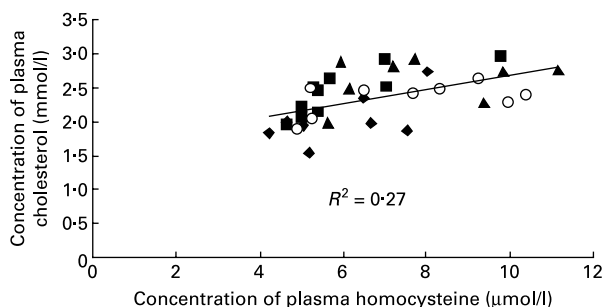For details of diets and procedures, see p. 880.

Fig. 1. Correlation between concentrations of plasma homocysteine and plasma cholesterol in growing rats fed diets that contained different amounts of methionine ($P < 0.01$). The coefficient of determination (R^2) between plasma homocysteine and plasma cholesterol was 0.30. The regression equation is $Y = 1.66 + 0.106 \, X$ (Y, plasma cholesterol concentration, mmol/l; X, plasma homocysteine concentration, μmol/l). (◆) 2.6 g/kg; (■) 3.5 g/kg; (▲) 4.5 g/kg; (○) 6.0 g/kg.

growth increases cholesterol concentrations in the plasma and liver. This is in agreement with several reports showing that methionine supplementation of a low-methionine diet, for example a soyabean protein diet, causes hypercholesterolaemia in rats (Oda *et al.* 1989; Tanaka & Sugano, 1989; Saeki *et al.* 1990; Sugiyama *et al.* 1996, 1997).
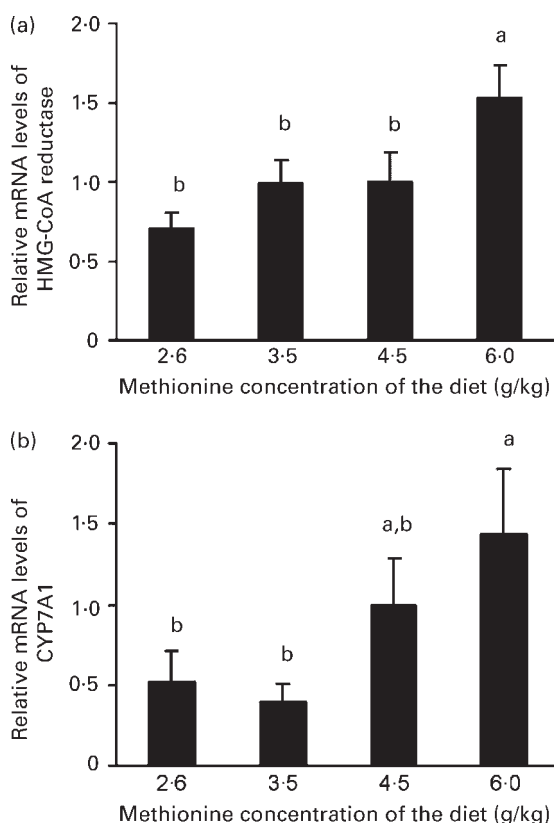

Fig. 2. (a) 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mRNA, and (b) cholesterol-7α-hydroxylase (CYP7A1) mRNA in the liver of growing rats fed 2.6, 3.5, 4.5 or 6.0 g methionine per kg diet for 14 d. Bars represent the means with their standard errors for ten rats. The mRNA concentrations of the rats fed 4.5 g/kg methionine were defined as 1, and the mRNA concentrations of the other groups were ascertained in relation to the mRNA concentrations of the group fed 4.5 g/kg methionine. [a,b,c] Mean values with unlike superscript letters were significantly different ($P < 0.05$). For details of diets and procedures, see p. 880.

In the present study, we observed that a moderate excess of dietary methionine increased the gene expression of HMG-CoA reductase in the liver of rats. Moreover, we observed that primary rat hepatocytes incubated with media supplemented with 100 or 200 μmol/l methionine had a higher cholesterol synthesis than cells incubated with a control medium that was supplemented with 50 μmol/l methionine. These observations suggest that methionine is able to stimulate cholesterol synthesis in liver cells. For technical reasons, we did not measure *in vivo* cholesterol synthesis in the rats. Nevertheless, the finding of elevated HMG-CoA reductase MRN levels in rat liver and of increased *in vitro* cholesterol synthesis in rat hepatocytes suggests that dietary methionine could have a hypercholesterolaemic action that is at least in part due to increased hepatic cholesterol synthesis.

The rat study was performed without any supplementation of exogenous cholesterol. Cholesterol is known to be an effective feedback inhibitor of HMG-CoA reductase, the key enzyme in endogenous cholesterol synthesis (Ott & Lachance, 1981). The observed effect of a moderate methionine excess on the expression of HMG-CoA reductase is therefore representative only for the experimental conditions used, but would possibly be modified by different dietary conditions, such as dietary cholesterol. The observed stimulation of HMG-CoA reductase expression in the high-methionine group is in agreement with observations by Woo *et al.* (2005), who showed that the mRNA concentration and activity of HMG-CoA reductase were significantly increased in the livers of rats fed a high-methionine diet with 17 g/kg methionine. In that study, homocysteine was suggested to be a stimulator of cholesterol synthesis in the liver via activation of the transcription factor SREBP-2 (Woo *et al.* 2005). We observed that the concentration of homocysteine in the media of primary rat hepatocytes markedly increased with increasing amounts of methionine added to the media. It therefore seems possible that homocysteine could have stimulated cholesterol synthesis in primary hepatocytes. However, the fact that there was only a weak positive correlation between the plasma concentrations of homocysteine and cholesterol in the rats fed diets with different methionine concentrations, together with the observation that plasma homocysteine concentrations were not significantly different between the rats fed diets with methionine concentrations between 2.6 and 6.0 g/kg, did not fully support this hypothesis. Thus, it is doubtful whether homocysteine by itself was responsible for the observed alterations in cholesterol metabolism.

However, an increase in plasma and liver cholesterol concentration was not only observed in the group fed 6.0 g/kg methionine but also in the groups fed 3.5 and 4.5 g/kg methionine compared with the group fed the low-methionine diet, although the relative mRNA concentrations of HMG-CoA reductase were not increased in these groups. It is fairly likely, therefore, that methionine influenced plasma and liver cholesterol concentrations not only by increasing the rate of cholesterol synthesis, but also by other mechanisms. Moreover, differences in the LDL receptor pathways do not seem to be responsible for the hypercholesterolaemic effect of dietary methionine because neither LDL receptor expression in the rats nor the uptake of LDL into cultivated hepatocytes was affected by methionine supplementation. We therefore cannot confirm the proposal that dietary methionine has a

Table 4. Phosphatidylcholine:phosphatidylethanolamine (PC:PE) ratio, rate of cholesterol synthesis, LDL uptake in primary hepatocytes, and methionine and homocysteine concentrations in the medium after incubation in media supplemented with 50, 100 or 200 µmol/l methionine for 24 h

(Mean values with their standard errors*)

Methionine supplementation (µmol/l)	50		100		200	
	Mean	SEM	Mean	SEM	Mean	SEM
PC:PE ratio (mol/mol)	3·14	0·51	3·14	0·43	3·48	0·51
Cholesterol synthesis (dpm/µg cell protein per 4 h)	7·3	1·9[b]	12·5	2·8[a]	12·8	2·4[a]
LDL uptake (ng LDL-protein/mg cell protein per 2 h)	176	27	192	28	200	43
Methionine (µmol/l)	45·8	3·5[c]	81·5	2·0[b]	138	21[a]
Homocysteine (µmol/l)	11·0	3·5[c]	16·8	1·7[b]	37·2	2·4[a]

*Six rats for PC:PE ratio and cholesterol synthesis; five rats for LDL uptake; three rats for methionine and homocysteine in medium.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).
For details of diets and procedures, see p. 880.

hypercholesterolaemic action by downregulating LDL receptors (Sugiyama *et al.* 1998).

The increased plasma cholesterol concentration in rats fed methionine-supplemented diets in the present study was mainly caused by an increase in circulating lipoprotein fractions with densities of over 1·063 kg/l and densities between 1·006 and 1·063 kg/l. The cut-off point chosen for lipoprotein separation was typical for human subjects but matched that used in previous rat studies (Geelen & Beynen, 2000; Giudetti *et al.* 2003). This means that the rat lipoprotein fraction with a density of $\rho > 1\cdot063$ kg/l contained only HDL, whereas the $1\cdot006 < \rho < 1\cdot063$ lipoprotein fraction also included intermediate-density lipoproteins, LDL and some HDL particles. Adding methionine to a low-methionine diet therefore mainly increased HDL, which normally promotes the transfer of peripheral free cholesterol to the liver by a mechanism known as 'reverse cholesterol transfer'. A methionine-induced increase in LDL and intermediate-density lipoprotein concentration could not, however, be ruled out. The mechanism responsible for the strong effect of methionine on HDL-cholesterol is not yet clear from the data analysed.

It is known that an increase in homocysteine concentration raises the level of homocysteine thiolactone, which can react with proteins by a mechanism involving the homocysteinylation of protein lysine residues, a process that damages proteins (Jakubowski, 2000*a*). Paraoxogenase is an enzyme that protects proteins against homocysteinylation by detoxifying homocysteine thiolactone (Jakubowski, 2000*b*) and is associated with the HDL fraction of serum lipoproteins (Jakubowski, 2000*a*). A recent study has shown that paraoxogenase activity, which is responsible for the detoxification of homocysteine thiolactone, correlates positively with the concentration of HDL cholesterol (Rozek *et al.* 2005). It would appear, therefore, that a link between the HDL-cholesterol concentration and the homocysteine concentration in this study is conceivable.

Another factor involved in cholesterol homeostasis is the excretion of cholesterol via bile acids. The conversion of cholesterol to 7α-hydroxylated bile acids is the principal pathway of cholesterol disposal. CYP7A1 is the initial and rate-limiting enzyme in the 'classic' pathway of bile acid synthesis, the major pathway for the elimination of cholesterol from the body. However, the concentrations of the relative mRNA of CYP7A1 were not indicative of diminished cholesterol excretion in the methionine-supplemented rats because the livers of rats fed the high-methionine diet showed a higher CYP7A1 expression rate than the livers of rats fed less methionine. In addition, the excretion of bile acids was no different between the groups fed low or high amounts of dietary methionine.

On the other hand, alterations in the methionine/homocysteine pathway are also believed to have a hypercholesterolaemic action by increasing the phosphatidylcholine:phosphatidylethanolamine ratio in the liver, which in turn raises the concentration of cholesterol in the plasma (Sugiyama *et al.* 1996, 1998; Giroux *et al.* 1999). The present finding of an increased hepatic phosphatidylcholine:phosphatidylethanolamine ratio in rats fed high-methionine diets confirms these results. However, the observation that hepatic PEMT gene expression was independent of the dietary methionine concentration suggests that the methionine-induced methylation of phosphatidylethanolamine was not regulated by the mRNA concentration of that enzyme. However, the fact that there was an *in vitro* stimulation of cholesterol synthesis when adding 100 instead of 50 µmol/l methionine to the cell culture media without any alteration in the phosphatidylcholine:phosphatidylethanolamine ratio suggests that the methionine-induced cholesterol synthesis was not primarily caused by altered phosphatidylcholine formation.

Another observation was that rats fed diets with a high methionine level exhibited an increased ratio of 20: 4*n*-6:18: 2*n*-6 in liver phosphatidylcholine. This suggests an increased activity of Δ6-desaturase, although the gene expression of that enzyme was not altered by dietary methionine. The activity of Δ6-deasturase, an integral protein of microsomal membranes, is strictly controlled by membrane fluidity (Brenner, 1981). Enrichment of the microsomal membranes with cholesterol lowers their fluidity and increases the activity of Δ6-desaturase (Garda & Brenner, 1985). Although we did not measure cholesterol in the microsomal membranes, we suspect that increased Δ6-desaturation in rats fed high-methionine diets may have been the result of the increased hepatic cholesterol concentrations observed in these animals.

In conclusion, this rat experiment confirms others that have shown that methionine supplementation of a low-methionine diet increases plasma cholesterol concentration in rats. The present study suggests that a moderate excess of dietary methionine may, at least in part, increase the concentration of plasma and liver cholesterol by stimulating cholesterol synthesis.

Acknowledgements

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