Hepatic very-low-density lipoprotein and apolipoprotein B production are increased following *in vivo* induction of betaine–homocysteine S-methyltransferase

Janet D. SPARKS^{*1}, Heidi L. COLLINS^{*}, Doru V. CHIRIEAC^{*}, Joanne CIANCI^{*}, Jenny JOKINEN^{*}, Mark P. SOWDEN[†], Chad A. GALLOWAY[†] and Charles E. SPARKS^{*}

^{*}Department of Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A., and [†]Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

We have previously reported a positive correlation between the expression of BHMT (betaine–homocysteine S-methyltransferase) and ApoB (apolipoprotein B) in rat hepatoma McA (McArdle RH-7777) cells [Sowden, Collins, Smith, Garrow, Sparks and Sparks (1999) *Biochem. J.* **341**, 639–645]. To examine whether a similar relationship occurs *in vivo*, hepatic BHMT expression was induced by feeding rats a Met (L-methionine)-restricted betaine-containing diet, and parameters of ApoB metabolism were evaluated. There were no generalized metabolic abnormalities associated with Met restriction for 7 days, as evidenced by control levels of serum glucose, ketones, alanine aminotransferase and L-homocysteine levels. Betaine plus the Met restriction resulted in lower serum insulin and non-esterified fatty acid levels. Betaine plus Met restriction induced hepatic BHMT 4-fold and ApoB mRNA 3-fold compared with Met restriction alone. No changes in percentage of edited ApoB mRNA were observed on the test diets. An increase in liver ApoB mRNA correlated with an 82 % and 46 % increase in ApoB and triacylglycerol production respectively using *in vivo* Triton WR 1339. Increased

secretion of VLDL (very-low-density lipoprotein) with Met restriction plus betaine was associated with a 45 % reduction in liver triacylglycerol compared with control. Nuclear run-off assays established that transcription of both *bhmt* and *apob* genes was also increased in Met-restricted plus betaine diets. No change in ApoB mRNA stability was detected in BHMT-transfected McA cells. Hepatic ApoB and BHMT mRNA levels were also increased by 1.8- and 3-fold respectively by betaine supplementation of Met-replete diets. Since dietary betaine increased ApoB mRNA, VLDL ApoB and triacylglycerol production and decreased hepatic triacylglycerol, results suggest that induction of *apob* transcription may provide a potential mechanism for mobilizing hepatic triacylglycerol by increasing ApoB available for VLDL assembly and secretion.

Key words: apolipoprotein B, betaine–homocysteine S-methyltransferase, 1-carbon metabolism, lipoprotein secretion, liver, very-low-density lipoprotein.

INTRODUCTION

Apolipoprotein B (ApoB) is expressed mainly in liver and intestine (reviewed in [1,2]). Its synthesis functions as an obligatory platform for the assembly of lipid by way of MTP (microsomal triacylglycerol transfer protein), and as a vehicle for secretion of hepatic triacylglycerol in the form of VLDL (very-low-density lipoprotein). Two forms of ApoB are synthesized: B100 and B48, the latter being related to the expression of APOBEC-1 (ApoB mRNA editing catalytic polypeptide-1) [3,4]. APOBEC-1, through a C-to-U editing reaction, introduces a stop codon in the ApoB mRNA, which terminates translation after 48 % of the protein has been synthesized. Numerous studies indicate that the *apob* gene is constitutively expressed and that the rate of ApoB secretion is determined by the amount of ApoB that escapes several presecretory degradation pathways [5]. However, several reports indicate that under some circumstances, transcriptional regulation may be involved in controlling the level of hepatic ApoB expression and secretion [6–10].

We recently identified a novel function of BHMT (betaine–homocysteine S-methyltransferase; EC 2.1.1.5) [11]. BHMT is a

45 kDa protein expressed mainly in liver and kidney of mammals [12], which catalyses the transfer of a methyl group from betaine to Hcy (L-homocysteine) forming dimethylglycine and Met (L-methionine), an essential amino acid [13]. When BHMT was expressed in McA (McArdle RH-7777) cells, ApoB mRNA abundance and secretion of ApoB-containing lipoproteins were substantially increased [11]. The effect of BHMT expression was specific, as levels of other mRNAs including apoA-I, apoE, albumin, MTP, β_2 -microglobulin, transferrin, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ubiquitin were unchanged.

In vivo, liver BHMT expression is influenced by dietary changes in sulphur amino acids, choline and betaine [14–16]. Previous studies indicated that Met restriction in a nutritionally adequate diet with betaine supplementation induces 8–10-fold increases in hepatic BHMT mRNA, immunodetectable BHMT protein and BHMT activity [17,18]. Using this model of BHMT induction as a nutritional parallel to BHMT overexpression in McA cells, we evaluated liver ApoB mRNA content and *in vivo* hepatic VLDL production. Results demonstrate that the relationship between BHMT expression and ApoB mRNA we observed in

Abbreviations used: ApoB, apolipoprotein B; APOBEC-1, ApoB mRNA editing catalytic polypeptide-1; BHMT, betaine–homocysteine S-methyltransferase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichlorobenzimidazole riboside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hcy, L-homocysteine; LDL, low-density lipoprotein; McA, McArdle RH-7777; Met, L-methionine; MTP, microsomal triacylglycerol transfer protein; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid; SAM, S-adenosylmethionine; SREBP, sterol-regulatory-element-binding protein; VLDL, very-low-density lipoprotein.

¹ To whom correspondence should be addressed (email Janet.Sparks@urmc.rochester.edu).

transfected McA cells is recapitulated *in vivo* through dietary BHMT induction, providing additional support for a physiologic linkage between 1-carbon metabolism and *apob* gene expression.

EXPERIMENTAL

Dietary treatments and animals

Experimental diets were prepared by Dyets (Bethlehem, PA, U.S.A.) based on the L-amino acid-defined AIN-93G diet containing (by weight): 18 % L-amino acids, 40 % starch, 14.5 % glucose, 10.0 % sucrose, 5 % cellulose and 7 % soya-bean oil. Dietary components were at levels recommended for the growing rat [19] except for the levels of Met, L-cystine, and choline bitartrate in the Met-restricted diet, which were at 1, 3 and 1.25 g/kg respectively. Choline levels, although lower than recommended, are sufficient to prevent the development of fatty liver when rats are fed a Met-restricted diet [20]. Betaine hydrochloride (Sigma, St. Louis, MO, U.S.A.) was added to the Met-restricted diet at a level of 3 g/kg (Met-restricted plus betaine) with the difference being made up by an equivalent decrease in microcrystalline cellulose. In some experiments, a control diet and a control diet plus betaine were used. The control diet was identical with the Met-restricted diet but containing 3 g/kg Met (Met-replete). The control diet plus betaine had adequate Met (3 g/kg) plus betaine (3 g/kg). All diets contained 10 g/kg of the antibiotic succinylsulphathiazole to inhibit microbial metabolism of methyl donors in the gastrointestinal tract, and were also supplemented with 50 mg/kg menadione (vitamin K). Unless otherwise indicated, male, 21-day-old Sprague–Dawley SD rats were used for dietary studies (Harlan, Indianapolis, IN, U.S.A.). All animal procedures were approved by the University Committee on Animal Resources, University of Rochester Medical Center.

Serum analyte measurements

Blood was collected and serum samples were prepared after allowing blood to clot on ice. Sera were stored frozen at -80°C until analysis. Serum cholesterol was quantified using a cholesterol oxidase method, and triacylglycerols were measured by assay of glycerol after release by lipase treatment, both being determined using commercial kits (Sigma Diagnostics). Serum ApoB was analysed by monoclonal RIA [21]. Serum glucose, NEFAs (non-esterified fatty acids) and ketones were determined by commercial kit assays (Glucose C2 kit, NEFA C kit and total ketone body kit; Wako Diagnostics, Richmond, VA, U.S.A.). Serum insulin was measured by RIA according to the manufacturer's instructions (Linco Research, St. Charles, MO, U.S.A.). Serum Hcy was measured using a fluorescence polarization immunoassay on the IMxAnalyzer (Abbott Laboratories, Oslo, Norway).

RNA isolation and analysis

Total RNA was isolated from a 0.5 g aliquot of liver according to the manufacturer's instructions. Purified RNA samples were dissolved in 200 μl of FORMAzol[®] (Molecular Research Center, Cincinnati, OH, U.S.A.), and an aliquot was removed to assess purity by measuring the ratio of absorbances at 260 and 280 nm, which were consistently greater than 1.9. For Northern blotting, liver RNA was separated on 0.8 or 1.2 % (w/v) agarose gels containing formaldehyde. Ratios of 28 S to 18 S RNA in liver RNA samples ranged from 1.8 to 2.0 as analysed on ethidium bromide-stained gels. Separated RNAs were transferred to Nytran Super Charge nylon membranes (Schleicher and Schuell Bioscience, Keene, NH, U.S.A.) using a turboblotter (Schleicher

and Schuell), and RNAs were immobilized by UV cross-linking [11]. Membranes were prehybridized in ExpressHyb[™] (BD Biosciences Clontech, Palo Alto, CA, U.S.A.), and then hybridized with ^{32}P -labelled cDNA probes [22]. cDNAs were labelled using Ready-To-Go[™] DNA labelling beads (minus dCTP) (Amersham Biosciences, Piscataway, NJ, U.S.A.) and [α - ^{32}P]dCTP (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA, U.S.A.). BHMT and ApoB probes used for Northern blotting were an EcoRI restriction fragment from BHMT cDNA (nt 866–1317) and a 206 bp PCR amplicon from rat ApoB (nt 6512–6719). Probes for GAPDH, cyclophilin, β -actin and 18 S RNA were obtained from Ambion (Austin, TX, U.S.A.). An Expressed Sequence Tag clone for mouse methionine synthase (GenBank[®] accession no. AA387056) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). After hybridization with labelled cDNAs, membranes were washed and evaluated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.) using ImageQuant[®] software. To control for gel loading, membranes were stripped by incubation in 0.5 % (w/v) SDS for 10 min at 100°C , and rehybridized with labelled GAPDH, cyclophilin or β -actin cDNA probes. The proportion of ApoB mRNA edited in rat liver was determined by RT (reverse transcriptase)–PCR methodology and PhosphorImager analysis as described previously [23].

RNase protection assay

The cDNA templates for synthesis of cRNA probes for rat SREBP-1 (sterol-regulatory-element-binding protein 1), SREBP-2 and β -actin were provided by Dr Joseph Goldstein (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) [24]. RNase protection assays were performed essentially as described previously [25]. Briefly, after linearization of plasmid DNA, antisense RNA probes were transcribed with [^{32}P]CTP (10 mCi/ml) with T7 RNA polymerase using the MAXIScript *in vitro* transcription kit (Ambion). Aliquots of total liver RNA (35 μg) were hybridized with cRNA probes for SREBP-1, SREBP-2 and β -actin using the RPA (ribonuclease protection assay) III kit (Ambion). After digestion with RNase A/T1, protected fragments were separated on 5 % (w/v) polyacrylamide/8 M urea gels. Protected fragments were quantified by PhosphorImager analysis, and corrected for differences for number of [^{32}P]CTP atoms as described in [24]. Signals for SREBP mRNAs were expressed relative to the β -actin signal.

In vivo lipoprotein production studies

Rats were fasted overnight to minimize the intestinal lipoprotein contribution to serum. In the morning, rats were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and then injected via the jugular vein with Tyloxapol (400 mg/kg; Triton WR 1339; Sigma) dissolved in saline (300 mg/ml) and warmed to 37°C . Blood was sampled from the jugular vein at zero time, and 1, 2 and 3 h, thereafter. Blood was allowed to clot at 4°C , and serum was prepared following centrifugation. Serum triacylglycerol and ApoB were measured at each time point, and production rates for individual rats were determined as the slope of the mass versus time curve using least-squared-fit analysis to determine the best line.

Liver lipid content

Frozen liver (300–350 mg) was homogenized in 15 ml of chloroform/methanol (2:1, v/v) to extract lipids using a Polytron tissue disrupter (medium speed for 15–30 s). Lipid extracts were filtered and residual tissue was rinsed with an additional 10 ml of

chloroform/methanol (2:1, v/v), which was filtered and combined with the first extract. Extracts were mixed with 5 ml of 0.15 M NaCl/0.05% (v/v) sulphuric acid and centrifuged at 1300 g to partition the chloroform layer. The chloroform layer was removed, evaporated under a stream of nitrogen, and lipid residues were dissolved in fresh chloroform and stored at -20°C in an explosion-proof freezer until analysis. Known volumes of extracts were mixed with 5% (v/v) Triton X-100/chloroform, and samples were aliquoted for lipid analysis as previously described [26,27]. After evaporation of chloroform, residues were directly solubilized in sample buffer, and analysed for cholesterol (Sigma) and for triacylglycerol content (L-Type TG H kit; Wako Chemicals USA).

Analysis of ApoB mRNA stability

McA cells stably transfected with empty vector (McA-CMV, where CMV is cytomegalovirus) or with rat BHMT (McA-BHMT) were cultured as previously described [11] in the presence of 500 $\mu\text{g/ml}$ Geneticin. After reaching 60–80% confluence in 60 mm dishes, cells were washed twice in 0.2% BSA/Hanks balanced salt solution, and cultured in DMEM (Dulbecco's modified Eagle's medium) containing 0.2% BSA and 100 μM DRB (5,6-dichlorobenzimidazole riboside) to arrest mRNA synthesis [28]. Cellular RNA was extracted using TRIzol[®] reagent 0, 3, 6, 9 and 12 h after addition of DRB, and ApoB mRNA levels were determined by Northern blotting. To control for gel loading, results were normalized to 18 S rRNA as RNA polymerase I-mediated transcription is not inhibited by DRB [28]. The half-life of ApoB mRNA was calculated by linear regression analysis of log-transformed decay curves.

Nuclear run-off analysis

Nuclear run-off assays were performed essentially as described in [29]. Rats were fed experimental diets for 7 days, and nuclei were isolated from whole liver homogenates, and stored frozen at -80°C in 0.2 ml aliquots in 50 mM Hepes (pH 8.0), 5 mM MgCl_2 , 0.5 mM dithiothreitol, 1 mg/ml acetylated BSA and 25% (v/v) glycerol. After thawing on ice and addition of 10 μl of RNasin plus (2 units/ μl ; Promega, Madison, WI, U.S.A.), nuclei were incubated with 1 mM each of CTP, ATP and GTP and 100 μCi of [α - ^{32}P]UTP (3000 Ci/mmol; New England Nuclear). Nascent ^{32}P -labelled RNA transcripts were isolated with TRIzol[®] reagent after DNase I and proteinase K treatments. After the final ethanol precipitation step, RNA was dissolved in 0.5% SDS, and was purified by MicroBiospin 30 (Bio-Rad) chromatography. Equal amounts of radioactive RNA from each nuclei preparation (3×10^6 c.p.m./ml ^{32}P -labelled RNA) were hybridized to linearized plasmid DNAs (5 μg of DNA/slot) previously blotted to Nytran membranes for 72 h at 42°C . Plasmids used were pcDNAIII containing a 2.9 kb cDNA from the 3'-end of rat ApoB sequence previously reported [30], pcDNAIII vector (for background correction), pSP73-BHMT (nt 866–1317) and pBluescript-GAPDH (316 bp). After extensive washing at 60°C and treatment of membranes at 37°C with RNases T1 and A, membranes were subjected to PhosphorImager analysis.

Statistical analysis

Unless otherwise indicated, results are expressed as mean values ± 1 S.D., where (n) is the number of individual samples analysed. Significance was determined using unpaired, two-tailed Student's t test.

Table 1 Effect of test diets on selected body, serum and liver parameters

Weanling rats were fed a Met-restricted diet, a Met-restricted diet plus betaine and a Met-replete Control diet (six rats per group) for 7 days, and a number of whole body, liver and serum parameters were measured. Results are expressed as means $\pm$ S.D.; i.u., international unit.

Animal parameters	Met-restricted	Met-restricted + betaine	Control
Final body weight (g)	50 $\pm$ 3.7*	51 $\pm$ 2.7*	88.4 $\pm$ 4.4
Liver weight (g)	2.02 $\pm$ 0.29*	2.23 $\pm$ 0.33*	5.07 $\pm$ 0.37
Liver (% body weight)	4.08 $\pm$ 0.51*	4.43 $\pm$ 0.41*	5.49 $\pm$ 0.48
Liver triacylglycerol (mg/g)	10.05 $\pm$ 4.09	7.39 $\pm$ 2.56*	13.54 $\pm$ 3.10
Liver cholesterol (mg/g)	8.28 $\pm$ 1.75	7.03 $\pm$ 1.44	6.59 $\pm$ 1.39
Serum metabolites and insulin levels			
Glucose (mg/dl)	185 $\pm$ 17	178 $\pm$ 24	179 $\pm$ 14
NEFAs (μM)	261 $\pm$ 70*	244 $\pm$ 88*	385 $\pm$ 90
Insulin (pM)	852 $\pm$ 121*	505 $\pm$ 140*	1467 $\pm$ 277
Ketones (μM)	372 $\pm$ 175	324 $\pm$ 129	358 $\pm$ 151
Alanine aminotransferase (i.u./l)	42 $\pm$ 11	41 $\pm$ 11	34 $\pm$ 10
Homocysteine (μM)	2.15 $\pm$ 0.87	2.46 $\pm$ 1.06	2.48 $\pm$ 0.72
Serum lipids and ApoB			
Triacylglycerol (mg/dl)	38 $\pm$ 17	30 $\pm$ 7	28 $\pm$ 9
Cholesterol (mg/dl)	69 $\pm$ 15	74 $\pm$ 8	70 $\pm$ 5
ApoB (mg/dl)	6.8 $\pm$ 1.3	7.3 $\pm$ 2.4	6.5 $\pm$ 2.0

* Indicates that means differ from that of rats fed the Control diet at a probability level of at least $P < 0.05$.

RESULTS

Growth characteristics of experimental rats

In order to examine the relationship between BHMT and ApoB expression *in vivo*, we chose a dietary manipulation known to induce high levels of hepatic BHMT [17,18]. When weanling rats are fed a Met-restricted diet containing adequate choline plus betaine, liver BHMT content and activity are markedly induced. Weanling rats were fed either the Met-restricted diet or Met-restricted diet plus betaine *ad libitum* for up to 14 days, and body and liver weights were monitored. Average weight gain of rats fed the Met-restricted diet was similar to that of rats fed the Met-restricted diet plus betaine (1.56 ± 0.57 g/day versus 1.61 ± 0.24 g/day respectively). This is a lower rate of growth compared with Met-replete diets, which has been attributed to less food intake and lower food efficiency [18]. Rats still gained weight, indicating that, although both diets are Met-restricted, they are above the minimum requirement for growth of weanling rats [18]. Liver as a percentage of body weight was relatively constant over the 14 days feeding period, and averages were similar on Met-restricted and Met-restricted plus betaine diets [$4.2\% \pm 0.41\%$ ($n = 15$) versus $4.3\% \pm 0.70\%$ ($n = 15$) respectively].

Metabolic status of rats on Met-restricted diets

To evaluate the possibility that the lower weight gain in Met-restricted diets resulted in an alteration of metabolism, we measured body, liver and serum parameters in rats fed the Met-restricted diets, and compared results with rats fed a Control diet (Met-replete) (Table 1). Body weights, liver weights and percentage of body weights were lower in the Met-restricted rats compared with control fed rats as has been previously demonstrated [17]. Liver triacylglycerols were significantly reduced in the Met-restricted diet plus betaine compared with Control or Met restriction alone. Insulin levels were reduced by Met restriction, but more so in the diet containing betaine. Serum-free fatty acid levels were also lower in the Met-restricted diets compared with the control. Glucose, total serum ketones, ApoB, cholesterol and triacylglycerol levels were comparable in the three diet groups. Together,

Figure 1 Time-dependent changes in steady-state levels of hepatic BHMT mRNA (A) and ApoB mRNA (B) in rats fed an amino acid-defined diet, restricted in Met (Diet A) or restricted in Met plus betaine (Diet B)

Initial weights (zero time) of rats in this experiment averaged 44 ± 2.9 g. After 3, 7 and 14 days on Diet A, rat weights averaged (four rats per group per time point): 46 ± 0.4 , 52 ± 6.4 and 66.2 ± 7.0 g. After 3, 7 and 14 days on Diet B, rat weights averaged (four rats per group per time point): 48 ± 2.1 , 57 ± 5.6 and 65 ± 1.2 g. Liver total RNA was isolated and levels of BHMT and ApoB mRNA were determined by Northern blotting. After stripping, blots were reprobed with 32 P-labelled cDNAs specific for β -actin and cyclophilin.

these results suggest that Met restriction itself did not produce global metabolic abnormalities, hepatic steatosis or altered liver function.

Effect of BHMT induction on ApoB mRNA abundance

The predicted changes in BHMT mRNA [17,18] were confirmed by Northern-blot analysis of liver RNA derived from rats fed the Met-restricted diet plus betaine for 3, 7 and 14 days (Figure 1A). There were marked increases in hepatic BHMT mRNA abundance compared with rats fed the Met-restricted diet. In corresponding samples, significant increases in hepatic ApoB mRNA levels were also observed in rats fed the Met-restricted plus betaine diet (Figure 1B). The average level of induction and variability of response were determined in three independent feeding experiments where rats were fed test diets for 3, 7 or 10 days (Table 2). The relative hepatic BHMT mRNA level in rats fed the Met-restricted plus betaine diet was 4.1–5 times greater than that in rats fed the Met-restricted diet. ApoB mRNA abundance was between 2.7 and 4.2 times greater in rats fed the Met-restricted diet plus betaine compared with rats fed the Met-restricted diet alone. Results demonstrate that feeding the Met-restricted diet plus betaine increased BHMT mRNA expression 4-fold and ApoB mRNA 3-fold compared with Met restriction alone, and both had a similar time course of induction. Because 7 days on diet produced maximal changes in ApoB and BHMT mRNA levels, subsequent feeding studies were carried out for 7 days.

Temporal changes in hepatic BHMT and ApoB mRNA levels

In a separate study, we compared hepatic ApoB and BHMT mRNA levels at the beginning of the feeding period with those

Table 2 Time course of diet-induced changes in hepatic BHMT and ApoB mRNA abundance

Rats were fed a Met-restricted diet or a Met-restricted diet plus betaine for 3, 7 and 10 days. At the end of the feeding period, total liver RNA was isolated, and levels of BHMT, ApoB and GAPDH mRNAs were analysed by Northern blotting. BHMT and ApoB mRNA contents were normalized to GAPDH mRNA. RATIO refers to the normalized signal ratio of the Met-restricted plus betaine-fed rats divided by that of the Met-restricted fed rats. Results are the average probe-to-GAPDH signal ratio ($\times 1000$) in three independent feeding experiments $\pm$ S.E.M., where three to six livers in each dietary group in each experiment were analysed.

Days	BHMT relative to GAPDH ($\times 1000$)			ApoB relative to GAPDH ($\times 1000$)		
	Met-restricted	Met-restricted + betaine	RATIO	Met-restricted + betaine	RATIO	Met-restricted
3	1037 ± 82	$4209 \pm 309^*$	4.1 ± 0.17	30 ± 3	$80 \pm 8^*$	2.7 ± 0.26
7	1236 ± 168	$5338 \pm 1107^*$	4.3 ± 0.41	37 ± 9	$141 \pm 38^*$	4.2 ± 1.12
10	1207 ± 203	$6181 \pm 1515^*$	5.0 ± 0.67	36 ± 7	$145 \pm 54^*$	4.0 ± 1.03

* Indicates a significant difference between the Met-restricted and Met-restricted plus betaine diets at a probability level of at least $P < 0.05$.

after 7 days on diet, calculating results similar to those described in Table 2. The relative hepatic ApoB mRNA abundance in Met-restricted rats at 7 days was similar to that observed at the initiation of the feeding period [39 ± 7 (6) versus 54 ± 14 (6), n being in parentheses]. Relative hepatic BHMT mRNA abundance in Met-restricted rats at 7 days was increased relative to that observed initially [1702 ± 121 (6) versus 897 ± 91 (6), $P < 0.05$]. This result shows the small effect of Met restriction alone on increasing BHMT mRNA levels as previously reported [18]. In marked contrast with rats fed the Met-restricted diet, Met restriction plus betaine produced marked increases in ApoB and BHMT mRNA levels compared with initial levels. By 7 days, ApoB mRNA levels averaged 197 ± 45 (6), and BHMT mRNA levels averaged 8606 ± 475 (6). No significant differences were observed in percentage of edited ApoB mRNA among the three diet groups [Met-restricted averaged $42.5\% \pm 6.3\%$ (3); Met-restricted plus betaine averaged $38.8\% \pm 9.2\%$ (3); Control averaged $44.4\% \pm 7.6\%$ (3)]. Since a 1-fold increase in BHMT mRNA by Met restriction alone did not result in significant changes in ApoB mRNA, whereas an 8-fold increase did, the results suggest that a critical level of BHMT induction may be required to observe changes in liver ApoB mRNA content.

Specificity of mRNA increases with rats fed betaine

To assess whether the observed changes in mRNA levels in rats fed Met-restricted plus betaine reflected a general increase in liver mRNAs, we analysed a number of additional mRNAs by Northern blotting. In livers derived from rats fed the Met-restricted or the Met-restricted plus betaine diets for 7 days, the probe-to-GAPDH signal ratio ($\times 1000$) averaged, for cyclophilin mRNA, 594 ± 38 versus 683 ± 42 ($n = 10$); for apoA-I mRNA, 3624 ± 318 versus 4342 ± 564 ($n = 9$); for apoE mRNA, 5955 ± 909 versus 5598 ± 1038 ($n = 6$); and for methionine synthase mRNA, 16.1 ± 2.0 versus 13.6 ± 0.8 ($n = 3$). As no significant differences were observed in these mRNAs, results suggest that there is relative specificity for the changes in hepatic ApoB mRNA abundance with BHMT induction. This level of specificity was also observed in previous studies of McA cells expressing high levels of BHMT [11].

Developmental age and dietary effects on ApoB mRNA

The rat *apob* gene is developmentally regulated [31,32], and liver ApoB mRNA levels fluctuate after birth, rising through the

Table 3 Changes in expression of SREBP mRNAs in rats fed Met-restricted, Met-restricted plus betaine and Control diets

Rats were fed a Met-restricted diet, a Met-restricted diet plus betaine or a Met-replete Control diet for 7 days. At the end of the feeding period, liver RNA was isolated, and levels of SREBPs were evaluated by RNase protection assay. Results are the average expression level relative to β -actin ($\times 100$), where (n) is the number of individual rat livers analysed.

mRNA	Met-restricted	Met-restricted + betaine	Control
SREBP-1a	30 $\pm$ 4 (7)	38 $\pm$ 5 (5)	27 $\pm$ 4 (7)
SREBP-1c	417 $\pm$ 54 (5)*	387 $\pm$ 50 (5)*	195 $\pm$ 17 (7)
SREBP-2	241 $\pm$ 25 (5)	283 $\pm$ 24 (5)*	189 $\pm$ 16 (7)

*Indicates significant differences between Met-restricted diets and the Control diet at a probability level of at least $P < 0.05$.

postnatal period until reaching adult levels by 5–6 weeks of age. It is therefore possible, since we used weanling rats, that dietary betaine may have accelerated developmental increases in liver ApoB mRNA to adult levels compared with rats fed Met-restricted diets alone. To address this issue, a control study was performed using 7-week-old rats when hepatic ApoB mRNA levels have stabilized to adult levels [32]. Rats were fed Met-restricted or Met-restricted plus betaine diets, and hepatic ApoB and BHMT mRNA levels were evaluated by Northern blotting. In Met-restricted and Met-restricted plus betaine diets, liver weights (7.71 ± 1.64 g versus 7.00 ± 1.44 g), body weights (167 ± 13 g versus 149 ± 15 g), and livers as a percentage of body weight (4.6 ± 0.75 % versus 4.7 ± 0.77 %) were similar. Compared with Met restriction alone, liver ApoB mRNA was increased 1.9 times ($P < 0.02$), and BHMT mRNA was increased 2.9 times ($P < 0.01$) by the betaine diet. Results demonstrate that in rats with adult levels of ApoB mRNA, ApoB mRNA remained responsive to betaine induction, suggesting that the effect was not due to accelerated developmental increases.

In vivo VLDL production

To address whether the increase in hepatic ApoB mRNA abundance that occurs with dietary BHMT induction leads to increases in hepatic secretion of ApoB-containing lipoproteins, we measured *in vivo* VLDL production rates. Triton WR 1339 injection was used to block the catabolism of nascent particles, and the incremental increase in serum triacylglycerol and ApoB over time was used to estimate hepatic VLDL production rate. Rats fed the Met-restricted plus betaine diets secreted 82 % more ApoB ($P < 0.001$) than rats fed the Met-restricted diet [$82 \pm 21.4 \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$ ($n = 7$) versus $45 \pm 10.8 \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$ ($n = 7$)], and 46 % more triacylglycerol ($P < 0.05$) [$44.2 \pm 4.52 \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$ versus $30.3 \pm 9.65 \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$ ($n = 7$)]. Thus the increase in hepatic ApoB mRNA content observed with dietary betaine was consistent with an increase in ApoB available for assembly with lipid and enhanced lipoprotein secretion.

Dietary effects on SREBP mRNA expression levels

The increased hepatic VLDL production in rats fed the Met-restricted plus betaine diet did not lead to increased serum ApoB or triacylglycerol levels (Table 1). To gain insight into possible mechanisms for this finding, we analysed hepatic expression of SREBP mRNA by RNase protection assay (Table 3). SREBPs are transcription factors that are responsible for the expression of key enzymes in fatty acid and cholesterol biosynthesis as well as in lipoprotein degradation pathways [33]. Met restriction alone doubled the levels of SREBP-1c compared with the Control diet.

Figure 2 Decay of ApoB mRNA in McA cells stably transfected with empty vector (McA-CMV) or plasmid containing BHMT (McA-BHMT)

McA cells were cultured in cDMEM (complete DMEM, which contains 10 % v/v, horse serum and 10 % v/v, fetal calf serum), and then incubated with $100 \mu\text{M}$ DRB. Total RNA was isolated at various times after DRB addition, and ApoB mRNA abundance was determined by Northern blotting (A). After stripping, blots were reprobed with a ^{32}P -labelled cDNA probe for 18 S ribosomal RNA to correct for gel loading. Average ApoB mRNA levels (ApoB signal/18 S signal) from three independent experiments were plotted against time of incubation with DRB (B).

Interestingly, Met restriction plus betaine caused a 50 % increase in SREBP-2 mRNA compared with the Control diet. An important SREBP-2 target is the LDL (low-density lipoprotein) receptor [34]. Results suggest the possibility that increased hepatic VLDL production in response to the Met-restricted plus betaine diet is balanced with increased clearance of secreted lipoproteins via the LDL receptor.

Role of ApoB mRNA stabilization in ApoB mRNA changes

The changes observed in hepatic ApoB mRNA levels could be due to either mRNA stabilization or to increased *apob* gene transcription. To address whether mRNA stabilization was responsible, we proposed to examine ApoB mRNA decay in primary hepatocytes derived from rats fed the Met-restricted and Met-restricted plus betaine diets. Pilot studies, however, indicated that BHMT mRNA had a short half-life in primary hepatocyte cultures, averaging only 3.5 h. Because the half-life of ApoB mRNA is much longer than that of BHMT mRNA (16 h) [35], we elected to evaluate mRNA stability in McA cells stably expressing BHMT (McA-BHMT), which are known to maintain a high level of expression of both ApoB and BHMT mRNAs. McA-BHMT and McA cells transfected with empty vector (McA-CMV) were incubated with DRB, an mRNA transcriptional inhibitor [28]. At various times after DRB addition, cellular RNA was isolated, and ApoB, BHMT and 18 S ribosomal RNA were assessed by Northern blotting (Figure 2). The calculated average half-life of ApoB mRNA was 6.7 ± 0.6 h in McA-CMV versus 7.1 ± 0.7 h in McA-BHMT. Although ApoB mRNA half-life in McA cells is shorter than that reported for HepG2 cells, the similarity of half-lives of McA-CMV and McA-BHMT supports the idea that increased BHMT expression did not lead to stabilization of cellular ApoB mRNA.

Figure 3 Nuclear run-off assay of hepatic *bhmt* and *apob* genes

Nuclei were isolated from livers derived from rats fed on various diets for 7 days. Nascent ^{32}P -labelled transcripts were hybridized to specific cDNA probes immobilized on membranes, and analysed using the PhosphorImager. Each strip represents a nuclei preparation from an individual rat. Betaine-fed rats (rows 1–3), rats fed Diet C (rows 1 and 2) and rats fed Diet A (rows 3 and 4) are indicated.

Induction of ApoB gene transcription by dietary betaine

To determine whether the increased content of hepatic ApoB mRNA observed with rats fed the Met-restricted plus betaine diet was due to increased transcription of the *apob* gene, nuclear run-off assays were performed (Figure 3). Rats were fed the Met-restricted, Met-restricted plus betaine or Control diet, and nuclei were isolated from livers for run-off assay. Nuclei were incubated with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$, and nascent ^{32}P -labelled transcripts were isolated, hybridized to specific cDNA probes and visualized by PhosphorImager analysis. Levels of nascent BHMT and ApoB transcripts were similar in nuclei derived from rats fed the Control diet (rows 1 and 2) compared with nuclei derived from rats fed the Met-restricted diet (rows 3 and 4). However, nuclei derived from rats fed the Met-restricted diet plus betaine (rows 5 and 6) had significantly more labelled ApoB transcripts than control rats. Normalization to *gapdh* transcripts showed that *apob* gene transcription was significantly increased by Met restriction plus betaine. Normalized *bhmt* gene transcription was also significantly increased in livers derived from rats fed Met-restricted plus betaine diets compared with Control. Results support that the increased hepatic content of BHMT and ApoB mRNAs in rats fed the Met-restricted plus betaine diet is most likely due to increased transcription of their respective genes.

Effect of betaine on ApoB and BHMT mRNA levels in rats fed the Met-replete diet

To determine whether similar effects on ApoB and BHMT mRNA levels are observed in diets containing adequate Met levels, feeding studies were carried out using the Control, Met-replete diet with betaine supplementation. Studies are of interest because of the suggestion of employing dietary betaine as an agent to treat non-alcoholic fatty liver disease [36,37]. Weanling rats were fed the Control diet with and without betaine supplementation for 7 days, and afterwards, liver RNA was isolated and ApoB and BHMT mRNA levels were assayed by Northern blotting (Figure 4). Compared with Control diet, dietary betaine increased BHMT mRNA expression 3-fold [696 ± 133 (5) versus 2770 ± 483 (5)]. ApoB mRNA levels were also significantly increased in Control plus betaine diets with an average increase of 1.8-fold [15 ± 2 (5) versus 43 ± 10 (5)]. These results indicate

Figure 4 Northern-blot analysis of ApoB mRNA and BHMT mRNA in livers derived from rats fed Control diet (Met-replete) or Control diet supplemented with betaine (3 g/kg diet)

Liver total RNA was isolated and levels of BHMT and ApoB mRNA were determined by Northern blotting. After stripping, blots were reprobed with ^{32}P -labelled cDNA specific for GAPDH mRNA. Each lane represents results from an individual rat liver.

that Met restriction is not necessary for the induction of ApoB mRNA resulting from betaine supplementation.

DISCUSSION

Levels of ApoB mRNA and lipoprotein secretion are specifically increased in McA cells following stable expression of BHMT [11]. In order to examine the effect of BHMT on ApoB RNA and protein in a more physiological context, we used an *in vivo* nutritional model known to induce liver BHMT expression. This model involves feeding weanling rats a Met-restricted, amino acid-defined diet, supplemented with betaine [17,18]. Rats fed the betaine-supplemented diet demonstrated significant increases in liver ApoB mRNA content that was maintained over a 14-day period. The increase in ApoB mRNA was relatively specific as no discernible differences were observed in mRNAs for cyclophilin, apoA-I, apoE or methionine synthase. A similar degree of specificity was observed in BHMT-transfected McA cells [11]. The increased levels of hepatic ApoB mRNA were associated with increased production of VLDL ApoB and triacylglycerol *in vivo*, suggesting that betaine increased the availability of ApoB for lipoprotein synthesis and secretion. This would be consistent with the reduction in hepatic triacylglycerol content observed in betaine-fed rats. Considering that serum triacylglycerol and ApoB concentrations were comparable across diet groups, our results suggest that increased levels of VLDL triacylglycerol and ApoB secretion were coupled with increased lipoprotein clearance. This hypothesis is consistent with the finding of elevated hepatic SREBP-2 mRNA, whose gene product is a key regulator of LDL receptor expression. A similar situation is observed in transgenic mice expressing nuclear SREBP, where high levels of cholesterol and fatty acid synthesis in liver are not associated with elevated plasma lipid levels as VLDL is rapidly removed from the circulation through the action of LDL receptors [34,38,39].

Although rats fed the Met-restricted diet gained weight more slowly than rats fed the Met-replete diet, no major metabolic disturbances were observed as evaluated by body, serum and liver parameters. Rats fed Met-restricted diets were not hypoglycaemic, ketotic, hyperhomocysteinaemic or hyperlipoproteinaemic. Moreover, there was no significant hepatic steatosis or liver function abnormality compared with age-matched rats fed Met-replete diets. This is in sharp contrast with the major metabolic disturbances in 1-carbon metabolism observed in rodents fed Met-void and choline-void diets, and ethanol diets that characteristically produce hepatic steatosis [40].

Changes in ApoB secretion are thought to occur primarily as a consequence of post-transcriptional mechanisms, as the *apob*

gene is considered to be constitutively expressed. Although a number of studies have demonstrated that ApoB mRNA content can vary in response to certain stimuli *in vitro* in HepG2 cells [7,8,10], rat hepatocytes [41] and CaCo-2 cells [10,42,43], and *in vivo* [6], reported changes are relatively small. In the present study, we demonstrate substantial increases in *apob* gene transcription induced by dietary betaine. Hepatic *apob* gene expression is regulated by a number of proteins that interact with enhancer and reducer elements present in the proximal promoter region, the second intron and the 5'-distal portion of the ApoB gene (reviewed in [44–46]). A region of chromosome 6 encompassing exons 1–8 of rat *apob* and including 1.5 kb upstream of the probable transcription start site was identified by a BLAST alignment of the human *apob* promoter [44] with the rat and mouse genome assemblies. Several potential transcription factor-binding sites were identified including core promoter elements for TBP (TATA box binding protein), SP1 (*trans*-acting transcription factor 1) and C/EBP α (CCAAT/enhancer-binding protein α) as well as for regulatory proteins relevant to hepatic metabolism PPAR (peroxisome-proliferator-activated receptor), SREBP-1, HNF-3 (hepatocyte nuclear factor-3), NF-ODC-1 (nuclear factor ornithine decarboxylase-1) and glucocorticoid receptor. Which specific regulatory elements and transcription factors are involved in the enhancement of hepatic *apob* gene transcription by dietary betaine needs to be addressed in future studies.

The relationship between BHMT and ApoB was discovered by screening for ApoB mRNA-associated proteins using monoclonal antibodies against ApoB RNA editing 60 S pre-editosome fractions [11]. Expression of BHMT in McA cells did not change the proportion of edited ApoB mRNA, but increased ApoB mRNA abundance 2–3-fold, while increasing ApoB secretion by 50 % [47]. Similar results are observed in the present *in vivo* study where BHMT induction in Met-restricted diets was associated with a 3-fold increase in liver ApoB mRNA content without change in the proportion of edited mRNA and with an 82 % increase in hepatic ApoB production. The lack of correspondence between ApoB mRNA expression and ApoB secretion is likely to be the result of presecretory ApoB degradation that has been documented in numerous systems [1,2,5].

Recently, metabolite analysis and gene expression profiling were conducted in orotic acid-induced fatty livers, and results have correlated increased ApoB mRNA with elevated hepatic betaine levels and disturbances in 1-carbon metabolism [48]. As betaine content of the liver is increased by dietary betaine [49], the present study showing that dietary betaine is associated with increased hepatic ApoB mRNA is consistent with a relationship between hepatic betaine levels and *apob* gene expression. In rodents, betaine is produced by the oxidation of choline, and provides the methyl group for remethylation of Hcy to form Met via BHMT [13] and dimethylglycine. This reaction conserves Met for the formation of SAM (*S*-adenosylmethionine), the principal methyl group donor in the body, and an important regulator of liver function [50]. Humans lack choline oxidase [51], and therefore do not produce betaine. Therefore, in humans, remethylation of Hcy via the BHMT pathway is dependent on dietary betaine. The BHMT pathway, however, is operational in humans, as dietary betaine has been shown to reduce significantly plasma Hcy levels [52]. Met is also produced from Hcy by the ubiquitously expressed enzyme, methionine synthase (EC 2.1.1.13), that utilizes 5-methyltetrahydrofolate as methyl donor and vitamin B₁₂ as a cofactor [13]. In the subsequent reaction via methionine adenosyltransferase (EC 2.5.1.6), SAM is generated, which can then be utilized in the methylation of DNA, RNA and histones. SAM is also the methyl donor for the remethylation pathway of phosphatidylcholine synthesis via PEMT (phosphatidyl-

ethanolamine *N*-methyltransferase) [53]. Additionally, SAM serves as the precursor of glutathione through conversion into cysteine via the trans-sulphuration pathway [13]. Whether the effect on *apob* gene expression we observed in the present study is related to hepatic betaine content, BHMT protein expression or SAM-dependent pathways is currently under investigation.

In vivo studies directly linking BHMT and ApoB expression are limited, but relationships from investigations focusing either on BHMT or on ApoB have been found with ethanol feeding. Ethanol affects 1-carbon metabolism by perturbation of both BHMT and methionine synthase pathways [54,55]. Methionine synthase has been shown to be inactivated by acetaldehyde, an ethanol breakdown product [56]. As a compensatory mechanism for the reduced activity of methionine synthase with ethanol feeding, there is an adaptive increase in liver BHMT that serves to maintain adequate levels of hepatic SAM [57]. Short-term ethanol treatment has been shown in separate studies to increase significantly ApoB mRNA content in HepG2 cells [8], and in rat hepatocytes [41], although other studies have not seen this relationship with chronic feeding [58]. Because high expression levels of BHMT in McA cells alone produced increases in ApoB mRNA similar to those observed by dietary induction of BHMT, current results point specifically to the importance of BHMT in this process.

Betaine feeding has been shown to decrease hepatic steatosis in ethanol-fed rats [59], and has recently been evaluated in pilot studies to treat humans with biopsy-proven NASH (non-alcoholic steatohepatitis) [37]. NASH occurs in a significant number of patients with obesity, diabetes and hyperlipidaemia and can lead to progressive fibrosis and cirrhosis (reviewed in [60]). Patients receiving betaine showed a marked improvement in degree of steatosis and in level of inflammation. These reports in the context of the present study suggest that dietary betaine could play a role in the mobilization of liver lipids. Whether mobilization of hepatic triacylglycerol in betaine-treated NASH patients involves increased hepatic expression of *apob* is not known. Alternative mechanisms could involve increased production of SAM that may favour lipoprotein lipid assembly via stimulated phosphatidylcholine synthesis. As SAM also serves as a precursor to glutathione, a major cellular antioxidant, increased hepatic glutathione may alleviate oxidative stress, and counteract peroxidation of polyunsaturated fatty acids, which enhances ApoB degradation, reduces ApoB available for VLDL assembly and leads to triacylglycerol accumulation in liver [61]. There are few documented conditions resulting in increased VLDL output caused by regulation of *apob* gene transcription under pathophysiologically relevant *in vivo* conditions. However, induction of *apob* gene expression by betaine may explain the beneficial effects of betaine therapy for hepatic steatosis in humans.

In summary, results indicate that *in vivo* induction of BHMT in rats with betaine-enriched diets increases ApoB mRNA levels. This confirms and extends previous studies *in vitro* in McA-BHMT cells [11]. Increased abundance of ApoB mRNA results in enhanced secretion of triacylglycerol and ApoB-containing lipoproteins and reduces hepatic triacylglycerol levels. The mechanism for the higher levels of hepatic ApoB mRNA is an increase in *apob* gene transcription. Dietary betaine induction of *apob* gene expression *in vivo* provides a possible intervention for mobilizing hepatic triacylglycerol by increasing VLDL export under conditions of hepatic steatosis.

We gratefully acknowledge the help of Dr Amy L. Wagner [Laboratory of Cellular and Developmental Biology, NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases)/NIH (National Institutes of Health), Bethesda, MD, U.S.A.] with the animal feeding studies. We thank Dr Harold C. Smith (Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY, U.S.A.) for his expert advice on nuclei isolations.

We also thank Dr Timothy Garrow at the University of Illinois at Urbana for many helpful discussions. This study was supported by grants R01 HL65398 (J. D. S.) and R01 DK43739 (to Dr Harold Smith, which provided for payment to C. A. G. and M. P. S.) from the NIH.

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Received 12 December 2005; accepted 6 January 2006

Published as BJ Immediate Publication 6 January 2006, doi:10.1042/BJ20051966