Hepatic Acyl-Coenzyme A:Cholesterol Acyltransferase-2 Expression Is Decreased in Mice with Hyperhomocysteinemia1–3

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Abstract

Alterations in lipid metabolism may contribute to the pathology of hyperhomocysteinemia (HHcy). Our objective in this study was to test the hypothesis that HHcy is associated with changes in liver acyl CoA:cholesterol acyl transferase 2 (ACAT2) expression and cholesteryl esters (CE) in mice with HHcy. ACAT2 is encoded by Soat2 and functions to catalyze the esterification of cholesterol with acyl-CoA. Mice heterozygous for disruption of the cystathionine-β-synthase gene (Cbs +/−) and C57BL/6 mice (Cbs +/+) were fed a control diet or a diet high in L-methionine (8.60 g/kg) and low in folic acid (0.20 mg/kg) to induce HHcy (HH diet). Lower Soat2 mRNA (P < 0.05) and ACAT protein (P < 0.001) were found in liver from Cbs +/− mice fed the HH diet, with higher plasma total homocysteine concentrations, than Cbs +/+ mice fed the control diet (35.01 ± 5.6 vs. 2.21 ± 0.6 µmol/L, respectively). In silico searches identified a CpG-rich region in the 5′ portion of the Soat2 gene, which was differentially methylated (P < 0.05) in Cbs +/− mice fed the HH diet than in Cbs +/+ mice fed the control diet and was accompanied by higher (P < 0.05) B1 repeat element methylation, an indicator of global de novo methylation. These findings show altered methylation and expression of Soat2/ACAT2 in liver from mice with HHcy and suggest a role for changes in liver CE in the pathology of HHcy.  J. Nutr. 140: 231–237, 2010.

Introduction

An elevated circulating concentration of total homocysteine (tHcy)8 is associated with increased risk of cardiovascular disease (CVD) (1). Endothelial dysfunction is a key feature of hyperhomocysteinemia (HHcy) in humans and animal models (2,3). The molecular mechanisms contributing to HHcy-related endothelial dysfunction are not fully known but may involve several pathways, including alterations in lipid metabolism and gene-specific changes in DNA methylation. Homocysteine is metabolically linked to cellular methylation reactions and lipid metabolism through the methionine cycle. Within the cycle, methionine is converted to S-adenosylmethionine (AdoMet), which serves as a methyl donor for numerous methyl acceptors, including phospholipids, DNA, RNA, protein, histones, and neurotransmitters (4). S-adenosylhomocysteine (AdoHcy) is produced as a result of methyl donation and homocysteine is formed through the (reversible) liberation of adenosine from AdoHcy. We and others have shown that in HHcy, liver AdoHcy levels are high, resulting in a lower AdoMet:AdoHcy ratio, global DNA hypomethylation, and gene-specific changes in DNA methylation (5–9).

Hepatic lipid accumulation, increased hepatic cholesterol biosynthesis, and decreased plasma HDL cholesterol and apolipoprotein (apo) A-I concentrations have been reported in humans and animals with HHcy (10–15). We have recently shown that the reduced AdoMet:AdoHcy ratio in liver from mice with HHcy is accompanied by methylation silencing of the gene for Δ-6 desaturase (Fads2) (9), which catalyzes the first step in the desaturation of the dietary essential fatty acids, linoleic acid [18:2(n-6)] and linolenic acid [18:3(n-3)], to arachidonic acid [20:4(n-6)] and docosahexaenoic acid [22:6(n-3)], respectively. This was accompanied by lower levels of arachidonic acid [20:4(n-6)] and docosahexaenoic acid [22:6(n-3)] in liver...
phospholipids and suggests that alterations in liver fatty acid metabolism may contribute to the pathology of HHcy.

Liver fatty acids are also incorporated into cholesteryl esters (CE) and altered CE fatty acid composition may contribute to the pathology of CVD. Esterification of cholesterol with fatty acyl-CoA is accomplished by acyl-CoA:cholesterol acyltransferase 2 (ACAT2) in liver and small intestine and by ACAT1 in all other tissues (16). In liver, the CE produced by ACAT2 is assembled and secreted into apo B-containing lipoproteins (17,18). The fatty acyl composition of circulating CE may contribute to vascular pathologies. Studies in nonhuman pri-

Mice and experimental protocol
Mice heterozygous for targeted disruption of the cystathionine-β-synthase (Cbs) gene (Cbs +/−) (20), on a C57BL/6 background, and their wild-type C57BL/6 littermates (Cbs +/+ ) were used in the study and genotyped for the disrupted Cbs allele, Cbs26tm1unc, and wild-type Cbs allele as described (8). At weaning, Cbs +/− mice and Cbs +/+ mice were fed either a control diet (TD 05108, Harlan Teklad) or a diet to induce HHcy with 8.60 g/kg L-methionine, 0.20 mg/kg folic acid, and succinyl sulfathiazole (HH diet) (TD 00205, Harlan Teklad) for 3–12 wk (Table 1). The HH diet has been previously used to induce HHcy (7,8). To determine the independent effects of a high-methionine diet (HM) or a low-folate (LF) diet, a group of Cbs +/+ and Cbs +/− mice were fed a HM diet (8.60 g/kg L-methionine) with adequate folate levels (TD 06252, Harlan Teklad) or a LF diet (0.20 mg/kg folic acid and succinyl sulfathiazole) for 3 wk with the same amount of methionine as the control diet (4.60 g/kg methionine) (TD 06253, Harlan Teklad) from weaning. All diets were devoid of cholesterol and supplied 14% (control diet) and 17% (HH, HM, LF diets) of total energy from soybean oil, which contained 10.4% palmitic acid (16:0), 4.4% stearic acid (18:0), and 22.5% 18:1(n-9). The diet groups were as follows: +/+C (Cbs +/+ mice fed the control diet); +/+HH (Cbs +/+ mice fed the HH diet); +/+HM (Cbs +/+ mice fed the HM diet); +/+LF (Cbs +/+ mice fed the LF diet); +/−HH (Cbs +/− mice fed the HH diet); +/−HM (Cbs +/− mice fed the HM diet); and +/−LF (Cbs +/− mice fed the LF diet). At 6–15 wk of age, mice were anesthetized with 1% Avertin (2,2,2-tribromoethanol) (0.3 mL/10 g body weight intraperitoneal) and blood was collected by cardiac puncture into EDTA (final concentration 5 mmol/L). Blood was centrifuged at 3000 × g for 20 min at 4°C and plasma was collected, immediately flash-frozen in liquid nitrogen, and stored at −80°C until later analysis of tHcy. Samples of liver were flash-frozen in liquid nitrogen and stored at −80°C for later extraction of genomic DNA, RNA, lipids, and protein. The protocol was approved by the University of British Columbia Animal Care Committee.

Biochemical analyses
Plasma tHcy, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds, was measured using a modified HPLC method with fluorescence detection (21). Total lipids were extracted from total liver and liver microsomal fractions (prepared as described below) using the method of Folch et al. (22) and individual classes of lipids were separated using a Waters 2690 Alliance HPLC (Waters Limited). The separated lipid classes were detected and quantified by evaporative light scattering detection (Model 2000, Alltech, Mandel Scientific) as described previously (23). Fatty acids were quantified as their respective methyl esters by GLC using heptadecanoic acid (17:0) as the internal standard on a Varian 3400 GLC (Varian Canada) equipped with a flame ionization detector (23).

Quantification of mRNA. Total RNA was extracted from liver using the RNasea Mini kit (Qiagen) and included DNase I-treatment to remove contaminating genomic DNA. Scd1 and Soat2 mRNA levels were quantified by real-time PCR using the comparative Ct method (2−ΔΔCt) (24) of relative quantification and commercially available primers and TaqMan MGB probes (FAM-fluoresceine dye labeled) from Applied Biosystems, as described previously (9).

ACAT2 immunoblot
Relative levels of ACAT2 protein in liver microsomal fractions were quantified by immunoblot. Samples of liver were homogenized in a 225-mmol/L sucrose and 25-mmol/L Tris buffer at pH 7.8 containing 10 mmol/L glutathione, 0.5 mg/L leupeptin, and 2 mg/L apro tin. Homogenized samples were centrifuged at 2000 × g for 20 min to remove debris followed by centrifugation of the supernatant at 10,000 × g for 20 min to pellet mitochondrial and nuclear fractions. The supernatant was then centrifuged at 105,000 × g for 60 min to pellet microsomes. The microsomal pellets were resuspended in a buffer containing 100 mmol/L sucrose, 50 mmol/L potassium chloride, 40 mmol/L potassium dihydrogen phosphate, and 30 mmol/L EDTA at pH 7.2 and protein concentrations determined using the Bio-Rad protein assay kit (Bio-Rad) based on the method of Bradford (25). For the ACAT2 immunoblot, 25 μg of protein of microsomal fractions were resolved on 10% Tris-HCl SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Immunodetection was performed using a goat anti-human ACAT2 polyclonal antibody (Santa Cruz Biotech) followed by a donkey anti-goat IgG antibody conjugated to alkaline phosphatase. Membranes were then stripped and probed for β-actin using a goat anti-human β-actin polyclonal antibody (Santa Cruz Biotech). Protein bands were detected and densities quantified using the Western Lightning CDP-Star chemiluminescence reagent (Perkin Elmer) and a ChemiGenius® gel imaging system (Perkin Elmer).

Quantification of Soat2/Scd1p6 methylation
Genomic DNA was extracted from liver using the DNeasy kit and included RNase I treatment (Qiagen). Genomic DNA samples (0.5–1 μg) were bisulfite-treated using the EZ DNA Methylation kit (Cedarlane

TABLE 1 Composition of diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>HH</th>
<th>LF</th>
<th>HM</th>
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<tr>
<td>Casein, g/kg</td>
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<td>132</td>
<td>132</td>
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<td>AIN-93G mineral mix, g/kg</td>
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<td>35.0</td>
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<td>Choline bitartrate, g/kg</td>
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<td>Folic acid, g/kg</td>
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<td>Cobalamin, μg/kg</td>
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<td>Riboflavin, mg/kg</td>
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<td>Succinyl sulfathiazole, g/kg</td>
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1 The diets contained no cholesterol and 14% (control, LF, HM) and 17% (HH) energy from fat.
2 Vitamin concentrations for these diets were from the AIN-93G vitamin mix (47), with each diet containing 10.0 mg/kg plus an additional 2.00 mg/kg of folic acid.
3 The HH and LF diets contained minimal levels of folic acid and 5.00 mg/kg succinyl sulfathiazole to prevent intestinal bacterial growth. All other vitamins (in addition to those listed) were added at the same concentration as in the AIN-93G vitamin mix (47).
4 Casein provides 4.60 g/kg methionine and 0.20 mg/kg folic acid.
Allele-specific methylation status of Soat2/Igfbp6

We cross-bred Cbs+/+ and Cbs+/- female mice with Cast/Ej (Cast) male mice and analyzed allele-specific methylation of Soat2/Igfbp6 in the F1 hybrid Cast × Cbs+/+ offspring fed the control diet and F1 hybrid Cast × Cbs+/- offspring mice fed the HD diet. The sequence of the CpG-rich region of Soat2/Igfbp6 was unknown for Cast mice, so we sequenced this region to find strain-specific variations that can be used for identification of parental alleles in the F1 hybrid offspring. For this we amplified the CpG-rich region of Soat2/Igfbp6 (Supplemental Fig. 1) of Cast mouse genomic DNA by PCR using the following primers: Soat2CF, 5'-GTTGATGGATATGGAGGTT-3' and Soat2CR, 5'-GGTGATGATATGTTGGTT-3'. Samples were analyzed in duplicate and the percent methylation at each CpG site was quantified using the Pyro Q-CpG software, version 1.0.9 (Biotage).

Statistical analysis

One-way ANOVA was used to compare findings in +/+C, +/+HH, +/+LF, +/+HM, and +/-HM mice. This was followed by the least significant difference test (if variances were equal) or the Games-Howell test (if variances were unequal) for multiple comparisons. Values presented are means ± SEM. These analyses were accomplished by SPSS for Windows version 12.0.1.
dietary 18:1(n-9) (3.2 and 3.9% total energy, respectively), we questioned whether the lower level of 18:1(n-9) in liver CE from mice with HHcy (Table 2) was the result of diminished endogenous synthesis of 18:1(n-9). Endogenous synthesis of 18:1(n-9) involves the desaturation of SFA by the enzyme Scd (33). HHcy did not affect liver Scd-1 mRNA levels (Fig. 1C). We also quantified total levels of fatty acids in liver to determine whether the overall pool of 18:1(n-9) was reduced in liver from mice with HHcy. In contrast to what we found for liver CE, total levels of 18:1(n-9) in liver were higher (P < 0.05) in +/-HH mice than in +/-HH and +/-C mice (Fig. 2A). This was accompanied by higher (P < 0.01) total levels of 16:0 in liver from +/-HH mice than +/-HH mice or +/-C mice (Fig. 2B). Total levels of 18:0 in liver were lower (P < 0.05) in +/-HH mice and +/-HH mice than in +/-C mice (Fig. 2C). Given that ACAT2 is found in the endoplasmic reticulum, we also quantified the levels of 18:1(n-9) in liver microsomal PC and PE and found no differences in liver microsomal PC 18:1(n-9) and significantly higher liver microsomal PE 18:1(n-9) in +/-HH mice than in +/-C and +/-HH mice (Fig. 2D).

The HH diet was high in dietary methionine (8.6 mg/kg) and low in dietary folic acid (0.2 mg/kg) to induce maximal increases in plasma tHcy. Others have shown that high dietary methionine intakes, independent of B vitamin status and HHcy, is proatherogenic in apo E knockout mice (34), raising the question of whether excess dietary methionine intakes, on their own and independent of HHcy, are pathological. We therefore determined if the effect of HHcy on liver Soat2 mRNA expression in our +/-HH mice was a consequence of elevated plasma tHcy and not the high methionine content of the HH diet. We found that, compared with +/-C mice, +/-LM mice and +/-HM mice had elevated plasma tHcy (10.6 ± 1.0 and 18.0 ± 2.1 μmol/L, respectively) accompanied by lower (P < 0.01) levels of liver Soat2 mRNA than +/-C mice (Table 3).

We further addressed the potential role for changes in DNA methylation in silencing Soat2 expression in mice with HHcy. We first assessed global de novo methylation status by quantifying the methylation status of 4 CpG sites within B1 repetitive elements (26,35). We found that +/-HH mice had higher (P < 0.05) mean methylation of the 4 CpG sites (by 23%) within B1 repetitive elements in liver than +/-C mice (Fig. 3). The most profound effect was observed at CpG 3, with +/-HH mice having higher (P < 0.05) methylation than +/-HH or +/-C mice (Fig. 3). Given these differences in global de novo methylation status in liver, we then determined whether the Soat2 gene was susceptible to HHcy-induced changes in DNA methylation. We found less (P < 0.05) methylation at CpG 6 and CpG11 in liver from 6-wk-old +/-HH mice than in +/-HH and +/-C mice (data not shown). We then investigated whether age and time of exposure to the HH diet influenced the methylation status of the Soat2 gene. Table 3 shows that the overall methylation status of the Soat2 gene was 39 and 28% greater (P < 0.01) in liver from 15-wk-old +/-C and +/-HH mice, respectively, than in 6-wk-old mice (data not shown).

The mouse Soat2 gene is located on chromosome 15 and the human SOAT2 gene on chromosome 12, both of which are in proximity to genomically imprinted genes, such as Slc38a4 and Igfbp6 (36,37). Given that allele-specific DNA methylation patterns play an important role in governing expression of genomically imprinted genes (38), we investigated whether there were differences in allele-specific methylation of Soat2 in liver from 6-wk-old +/-C and +/-HH mice. The methylation status of the maternal allele did not differ between the F1 hybrid Cast × +/-C mice with a plasma tHcy of 1.96 μmol/L and the F1 hybrid Cast × +/-HH mice with a plasma tHcy of 16.1 μmol/L (Fig. 4A). Conversely, we found that the F1 hybrid Cast × +/-HH had lower methylation of the paternal allele than the F1 hybrid cast × +/-C (Fig. 4B). These findings suggest that the effect of HHcy on the methylation status of the Soat2 gene is allele-specific, with the paternal allele more sensitive to the effects of HHcy.

Discussion

In this work, we sought to determine whether HHcy is accompanied by alterations in liver CE metabolism in mice and represents an extension of our recent findings that liver phospholipid and long-chain PUFA metabolism is altered in mice with HHcy (9). We postulate that the reduced methylation capacity in liver that accompanies HHcy creates disturbances in hepatic lipid metabolism, which contributes to the pathology of HHcy. Disturbances in lipid metabolism have been observed in humans and animals with HHcy and include hepatic lipid accumulation, increased hepatic cholesterol biosynthesis, and reduced plasma HDL cholesterol and apo A-I levels (10–15). There are 3 key findings of this study. The first finding is that mice with HHcy have reduced expression of Soat2 mRNA and ACAT2 protein accompanied by lower levels of 18:1(n-9) in liver CE despite higher total levels of 18:1(n-9) in liver. We also found that the effect of HHcy on Soat2 mRNA expression in Cbs +/- mice was independent of the dietary means by which HHcy
was achieved. The second finding is that we identified a CpG-rich region 5' to the Soat2 gene that was differentially methylated in liver from mice with HHcy. This was accompanied by higher B1 repetitive element methylation, an indicator of global de novo methylation. The 3rd finding is that the affect of HHcy on the methylation status of the Soat2 gene is allele specific, with greater methylation of the paternal allele in liver from mice with HHcy.

The effect of HHcy on liver CE fatty acid composition was greatest for the predominant monounsaturated fatty acids and SFA with 12% lower 18:1(n-9), 27% higher 18:0, and 7% higher 16:0 in liver CE from mice with HHcy. The only compositional differences between the experimental diets was in the amount of methionine and folic acid, with the lipid content almost identical, providing 14% (control diet) and 17% (HH diet) of total energy from soybean oil, and both were devoid of cholesterol. The effect of HHcy on liver CE fatty acid composition was only observed in the Cbs+/− mice fed the HH diet, who had the highest concentration of plasma tHcy, and was not observed in Cbs+/+ mice fed the HH diet with much lower plasma tHcy concentrations. The physiological relevance of the differences in liver CE fatty acids between mice with HHcy (+/HH mice) and the control mice (+/+C) remains to be determined. Studies in nonhuman primates have shown that accumulation of 18:1(n-9) in liver CE correlates with the degree of coronary atherosclerosis (19), highlighting the importance of liver CE fatty acid composition to the pathology of CVD. As such, we predicted that mice with HHcy would have elevated levels of 18:1(n-9) in liver CE rather then lower levels.

A probable explanation for the lower level of 18:1(n-9) in liver CE from mice with HHcy is decreased liver synthesis of cholesteryl oleate by ACAT2, because oleoyl CoA has been shown to be the preferred substrate for CE formation in isolated rat liver microsomes (39). The fatty acid composition of the experimental diets was nearly identical and we considered there may be decreased endogenous synthesis of liver 18:1(n-9) and/or decreased availability of 18:1(n-9) to serve as a substrate for the ACAT2 reaction. This is unlikely given we found higher levels of total 18:1(n-9) in liver. Also, Scd-1 mRNA expression in liver from mice with HHcy did not differ, suggesting there was no impairment in the synthesis of 18:1(n-9). However, the high variability within each group for Scd-1 mRNA levels makes it difficult to drawn conclusions from these findings.

<table>
<thead>
<tr>
<th>Cbs genotype/diet group</th>
<th>Plasma tHcy μmol/L</th>
<th>Liver Soat2 mRNA relative quantitation</th>
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</thead>
<tbody>
<tr>
<td>+/+C</td>
<td>2.50 ± 0.84</td>
<td>1.42 ± 0.17</td>
</tr>
<tr>
<td>+/+LF</td>
<td>6.96 ± 1.14</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td>+/− LF</td>
<td>10.6 ± 0.98</td>
<td>0.98 ± 0.10</td>
</tr>
<tr>
<td>+/− HM</td>
<td>6.20 ± 0.90</td>
<td>1.09 ± 0.13</td>
</tr>
<tr>
<td>+/− HM</td>
<td>18.0 ± 2.07</td>
<td>0.96 ± 0.10</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 6. Means in a column without a common letter differ, P < 0.01.

**FIGURE 2** Changes in total levels of oleic acid [18:1(n-9)] (A), palmitic acid (16:0) (B), and stearic acid (18:0) (C) in liver and levels of 18:1(n-9) (D) in liver microsomal PC and PE from +/+C, +/+HH, and +/−HH mice. Values are means ± SEM, n = 6–8. Means not sharing a common letter differ, P < 0.05.

**FIGURE 3** Changes in B1 repetitive element methylation status in liver from +/+C, +/+HH, and +/−HH mice. The methylation status of 4 CpG sites in the B1 repetitive element sequence were analyzed as an indicator of global de novo methylation (26,35). Values are means ± SE, n = 6. Means not sharing a common letter differ, P < 0.05.
The lower liver ACAT2 expression in mice fed the HH diet likely occurred because of HHcy and not the high methionine content of the diet, because we also observed lower liver Soat2 mRNA expression in Cbs +/− mice with HHcy induced by feeding the LF diet. Others have shown that high dietary methionine intakes, independent of B-vitamin status and plasma tHcy concentrations, is on its own pathological as it pertains to atherosclerosis (34). Most interestingly, it appears that the expression of ACAT2 is very sensitive to changes in plasma tHcy, as observed decreased liver ACAT2 expression in Cbs +/− mice fed the HH diet with only a mild elevation in plasma tHcy (6.8 ± 1.6 μmol/L). The physiological relevance of the decreased ACAT2 expression in mice with HHcy requires further investigation. Others have shown reduced hepatic apo B expression and decreased hepatic VLDL secretion in mice with severe HHcy (14). ACAT2, because of its role in the production of liver CE, plays an important role in governing liver secretion of apo B-containing lipoproteins (40). Therefore, our finding of reduced ACAT2 expression in mice with HHcy may contribute to the observation by others of reduced VLDL secretion in mice with HHcy.

We have previously shown that HHcy in mice is accompanied by lower AdoMet:AdoHcy ratios because of increased AdoHcy, and gene-specific changes in DNA methylation in liver (7–9). Furthermore, many reports have shown that HHcy is accompanied by global total DNA hypomethylation in human and mouse tissues (5–7, 41). In the current study, we found increased global de novo methylation status in liver of mice with HHcy, assessed by quantifying the methylation status of B1 repetitive elements (26, 35). De novo methylation is thought to be accomplished by DNA methyltransferase (DNMT) 3a and 3b, whereas maintenance methylation is thought to be accomplished by DNMT1 (42). Dnmt3a is expressed in mouse somatic tissues (43) and has recently been shown to play an important role in the methylation silencing of tumor necrosis factor α expression in human endotoxin tolerant THP-1 monocytes (44). Furthermore, others have shown that treatment of HUVEC cells with homocysteine is associated with decreased DNMT1 activity but has no effect on DNMT3 activity (45).

Most interestingly, we identified a CpG-rich region in the 5′ portion of the Soat2 gene that was differentially methylated in liver from mice with HHcy. The role for DNA methylation in regulating the expression of Soat2 is not known, but the changes in Soat2 methylation were accompanied by lower levels of Soat2 mRNA. In vitro studies have shown that transcriptional regulation of the human SOAT2 gene involves hepatic nuclear factor 1, which binds to a region of the SOAT2 promoter between −900 and −850 relative to the transcriptional start site (46). The differentially methylated region of the murine Soat2 gene identified in this study is located between −6032 and −5701 relative to the Soat2 transcriptional start site and is within the coding region of Igf2bp6.

In summary, the results of this study demonstrated that mice with HHcy have reduced expression of liver ACAT2 protein and Soat2 mRNA and lower levels of 18:1(n-9) in liver CE. We also observed increased global de novo methylation and altered allele-specific methylation of a CpG-rich region in the 5′ portion of Soat2 gene in liver from mice with HHcy. These findings suggest that mice with HHcy have impaired synthesis of liver cholesteryl oleate by ACAT2. Given these findings were accompanied by altered allele-specific methylation of a CpG-rich region in the 5′ portion of Soat2 gene, our future goal will be to determine a functional role for DNA methylation in governing liver Soat2 expression and the functional relevance of allele-specific differences in Soat2/Igf2bp6 methylation status.

Acknowledgments

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