Elevated Homocysteine Reduces Apolipoprotein A-I Expression in Hyperhomocysteinemic Mice and in Males With Coronary Artery Disease

Leonie G. Mikael, Jacques Genest Jr, Rima Rozen

Abstract—Hyperhomocysteinemia, a risk factor for cardiovascular disease, is caused by nutritional or genetic disturbances in homocysteine metabolism. A polymorphism in methylenetetrahydrofolate reductase (MTHFR) is the most common genetic cause of mild hyperhomocysteinemia. To examine mechanisms by which an elevation in plasma homocysteine leads to vascular disease, we first performed microarray analyses in livers of Mthfr-deficient mice and identified differentially expressed genes that are involved in lipid and cholesterol metabolism. Microarrays and RT-PCR showed decreased mRNA for apolipoprotein A (ApoA)-IV and for ApoA-I and increased mRNA for cholesterol 7α hydroxylase (Cyp7a1) in Mthfr<sup>−/−</sup> mice compared with Mthfr<sup>+/+</sup> mice. Western blotting revealed that ApoA-I protein levels in liver and plasma of Mthfr<sup>−/−</sup> mice were 52% and 62% of levels in the respective tissues of Mthfr<sup>+/+</sup> mice. We also performed Western analysis for plasma ApoA-I protein levels in 60 males with coronary artery disease and identified a significant (P<0.01) negative correlation (−0.33) between ApoA-I and plasma homocysteine levels. This cohort also displayed a negative correlation (−0.24, P=0.06) between high-density lipoprotein cholesterol and plasma homocysteine. Treatment of HepG2 cells with supraphysiological levels of 5 mmol/L homocysteine reduced peroxisome proliferator-activated receptor (PPAR) α and ApoA-I protein levels and decreased ApoA-I promoter activity. Transfection with a PPARα construct upregulated ApoA-I and MTHFR. Our results suggest that hyperhomocysteinemia may increase risk of atherosclerosis by decreasing expression of ApoA-I and increasing expression of CYP7A1. (Circ Res. 2006;98:564-571.)

Key Words: homocysteine □ methylenetetrahydrofolate reductase □ apolipoprotein A-I □ high-density lipoprotein □ peroxisome proliferator-activated receptor α
vascular cells, and increased cholesterol efflux from cells such as macrophages that are involved in the initial development of the atherosclerotic plaque.

A region on the long arm of chromosome 11q23-q24 encodes genes for 3 lipoproteins (apoA-I, apoC-III, and apoA-IV), which are clustered within a 17-kb segment. Comparative sequence analysis has identified a relatively new apolipoprotein, apoA-V, located approximately 30-kb proximal to the apoA-I-III-A-IV gene cluster.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors; 4 isotypes are known: PPARγ, PPARβ, PPARγ, and PPARδ. PPARγ, which is predominantly expressed in the liver, is involved in lipid metabolism and stimulates fatty acid oxidation and ketogenesis. The relevance of PPAR pathways to metabolic disease is underscored by the use of fibrates (PPAR agonists) to treat hyperlipidemia. PPARγ decreases triglyceride concentrations by increasing the expression of lipoprotein lipase, and increases HDL cholesterol levels by regulating the production of ApoA-I, ApoA-II, and ApoA-V. It also controls reverse cholesterol transport by increasing cholesterol efflux from macrophages and inhibits transcription of the gene encoding cholesterol 7α-hydroxylase (CYP7A1). Cyp7A1 is a liver-specific enzyme that catalyzes the 7α hydroxylation of cholesterol, the rate-limiting step in the conversion of cholesterol into bile acids. A negative correlation between high Hcy levels and PPAR expression has been suggested in humans. In a murine model of HHcy, PPARγ was shown to ameliorate endothelial dysfunction.

In this study, we used our Mthfr-deficient mouse model to study the potential interaction between homocysteine and lipid metabolism. Initial microarray findings of decreased apolipoprotein expression in liver of hyperhomocysteinemic mice were pursued by additional studies in murine tissues, in an effort to study the potential interaction between homocysteine and lipid metabolism. Initial microarray findings of decreased apolipoprotein expression in liver of hyperhomocysteinemic mice were pursued by additional studies in murine tissues, in a human cohort of coronary artery disease, and in HepG2 cells. Our findings suggest that HHcy may contribute to cardiovascular disease by decreased expression of ApoA-I and increased expression of CYP7A1, mediated by PPARγ.

Materials and Methods

Animal Model of Hyperhomocysteinemia

Mthfr-deficient mice (Mthfr<sup>−/−</sup>) and wild-type (Mthfr<sup>+/−</sup>) littermates were generated as reported. Mice were progeny from matings of Mthfr<sup>−/−</sup> mice backcrossed for at least 12 generations from 129/Sv-BALB/c F1 heterozygotes to BALB/cAnNCrlBR (Charles River, Saint-Constant, Quebec, Canada). Experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital. Mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred with C57Bl/6J mice to obtain heterozygous and wild-type genotypes. DNA was isolated using TRizol reagent (Invitrogen). Liver RNA from 3 pairs of Mthfr<sup>−/−</sup> mice and wild-type Mthfr<sup>+/−</sup> littermates was analyzed by Affymetrix GeneChip Expression Arrays at the McGill University/Genome Quebec Innovation Center (Montreal, Canada) using the murine chip MG-U74Av2; mice were matched for age (8 months) and gender (2 male and 1 female pair). Affymetrix software was used to assess genes with differential expression between wild-type and Mthfr-deficient mice, as described previously. For RT-PCR experiments, RNA from mouse liver was also DNase treated and reverse transcribed with random hexamers and SuperScript II as recommended by the manufacturer (Invitrogen). The cDNA was used in RT-PCRs with primers for mouse ApoA-I, ApoA-IV, Cyp7A1, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Quantitation of RT-PCR products was performed by measuring the intensity of each band using Quantity One 4.1.0 software and SPSS software for statistical analysis. Intensity of signal from the relevant genes was divided by the intensity of the signal from GAPDH, which served as control. To confirm RT-PCR results, quantitative real-time RT-PCR (qRT-PCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in a MX4000 QPCR System (Stratagene) following the recommendations of the manufacturers.

Preparation of Crude Liver Extracts

Frozen mouse liver was pulverized in liquid nitrogen and suspended in ice-cold radioimmunoprecipitation assay lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS [sodium dodecyl sulfate] in PBS) containing a protease inhibitor mixture (Complete Mini, Roche). After incubation on ice for 30 minutes, lysates were centrifuged (1500 rpm, 15 minutes, 4°C). Supernatants were collected and protein concentrations assessed.

SDS-PAGE and Western Blotting

Electrophoresis (50 µg of protein from liver extracts, 25 µg of protein from cultured cells, 1 µL of mouse or human plasma) was performed on SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes at 67 V for 2 hours at 4°C. Nonspecific binding sites were blocked with 5% skim milk powder in Tris-buffered saline/Tween buffer overnight at 4°C. Primary antibodies were: rabbit anti-mouse ApoA-I (Biodesign International), anti-human ApoA-I (Calbiochem), anti-β-actin (Sigma), anti-albumin (Bethyl Laboratories), and anti-PPARγ (described by Frost et al.). Secondary antibody was a peroxidase-coupled anti-rabbit IgG (Amersham Biosciences). Signal detection was achieved with ECL Plus chemiluminescence system (Amersham Biosciences) and exposure to x-ray films. Signals were quantified with Quantity One 4.1.0 software. SPSS software was used for statistical analysis.

Clinical Studies

The patient population consisted of a cohort of coronary artery disease patients from Montreal that had previously been characterized for homocysteine levels and other relevant parameters before treatment was initiated. Mean plasma tHcy concentration for the whole study cohort is 10.8 ± 5.0 µmol/L and an equal distribution above and below this mean, was 31 to 59 years, with a range of Hcy levels having a mean of 10.5 µmol/L and an equal distribution above and below this mean, and examined immunoreactive ApoA-I protein by Western blotting.

Plasmid Constructs for Human ApoA-I Promoter

The human ApoA-I promoter fragment was amplified from HepG2 DNA, between positions −330 and +69 relative to the transcription start site, using primers 5′-TATAGCTGAAGACACATGTGCAAGGCAACACTG-3′ and 5′-TTAAGGATCTTTGCCAGCT-GGGGAC-3′ that incorporated Nhel and HindIII restriction sites, as previously reported. The amplified fragment, which contained the peroxisome-proliferator receptor element (PPRE), was digested with Nhel and HindIII and inserted between the respective sites in...
pGL3-basic vector to create an ApoA-I wt plasmid. Mutations in the PPARE were generated by PCR using primers 5'-ACTGA-TCCCTGGTCCTGCTGACGCCCACGCA-3' and 5'-AGGGGAAAGGGCATCAGGGGGGCGGAGGAGGAT-3' (mutations underlined) to create the ApoA-I mut plasmid. Integrity of the inserts was verified by sequencing.

Plasmid Construct for Human PPARα

The human PPARα sequence was amplified from HepG2 cDNA and cloned into expression vector pCMV-Myc (BD Biosciences). After transformation into INVαF" Competent Escherichia coli cells (Invitrogen), potential clones carrying the human PPARα were screened and inserts from the positive clones were verified by sequencing.

HepG2 Cell Culture and Transient Transfection Assays

Human HepG2 hepatoma cells (American Type Culture Collection) were propagated at 37°C, in 5% CO2, in DMEM-F12 with 10% FBS, 100 µg/mL penicillin and streptomycin. For transfections, cells were seeded at approximately 5 × 10^5 into 6-well plates and medium was changed regularly. At 90% confluence, cells were transfected with either 2 µg of vector containing PPARα or pCMV vector alone using Lipofectamine 2000 Reagent in OPTI-MEM medium (Invitrogen) following the recommendations of the manufacturer. Cells were harvested 72 hours after transfection. RNA isolation, cDNA synthesis, RT-PCR, and q-RT-PCR were performed as above. Increased expression of PPARα was confirmed by RT-PCR in transfected cells. For Western blotting, cells were washed with ice-cold PBS and treated as described above. For transfection with ApoAI promoter-reporter plasmids, HepG2 cells were cotransfected with pCMV-β-galactosidase. Firefly luciferase activity was detected by a Luciferase Assay Kit (Promega); transfection efficiencies were controlled by normalizing to β-galactosidase activity (Applied Biosystems). Transfections were performed at least 4 times.

Homocysteine Treatment of HepG2 Cells

At 80% confluence, the medium was replaced with DMEM-F12 supplemented with 0, 1, 5, or 10 mmol/L of homocysteine or t-cysteine (Sigma). Cells were collected at specific time points for up to 24 hours, washed with ice-cold PBS and treated for Western blotting. Promoter activity was measured by luciferase activity. Hcy was added 6 hours after transfection by replacing OPTI-MEM with 0, 1, or 5 mmol/L Hcy in DMEM-F12; cells were harvested 24 hours after transfection.

Results

Effects of Mthfr Deficiency on ApoA-IV, ApoA-I, and Cyp7A1 mRNA Levels in Mouse Liver

The Affymetrix software algorithm was used to analyze microarray results and examine differentially expressed genes in 3 pairs of livers from Mthfr^−/− mice compared with Mthfr^+/+ littermates. We chose genes with a greater than 1.8-fold change (increase or decrease) and a difference of 150 U of fluorescence in the raw data analysis (Table I in the online data supplement available at http://circres.ahajournals.org). We were particularly interested in genes that were implicated in lipid and cholesterol metabolism and honed in on 2 such genes, ApoA-I and Cyp7A1. Mthfr^−/− mice had reduced expression of ApoA-IV compared with Mthfr^+/+ mice in all 3 pairs, with an average fold change of −2.6. Compared with their Mthfr^+/+ littermates, Mthfr^−/− mice showed increased expression of Cyp7A1 in all 3 pairs, with an average fold change of +2.1. Microarray analysis also revealed a decrease in ApoA-I mRNA levels in 2 pairs of Mthfr^−/− mice compared with their wild-type littermates; the fold change, however, did not reach statistical significance.

RT-PCR was performed to validate results of the microarray for the 3 genes of interest (Figure 1a). For quantitation, the signal intensity of each of the PCR products was measured and presented relative to GAPDH (internal control) (Figure 1b). The mean and standard deviation for mRNA levels in Mthfr^+/+ relative to Mthfr^−/− mice for ApoA-I were 0.71±0.07 in 4 pairs of mice (P<0.01); for ApoA-I, 0.61±0.2 in 4 pairs of mice (P=0.06); and for Cyp7A1, 1.5±0.1 in 3 pairs of mice (P<0.01). Results for ApoA-I were also examined by q-RT-PCR for 2 pairs of liver RNA from Mthfr^+/+ and Mthfr^−/− mice; these findings were confirmatory (Mthfr^+/+ values were 45±20% of those in Mthfr^−/−; data not shown).

Effects of Mthfr Deficiency on ApoA-I Protein Levels in Mouse Liver and Plasma

To determine whether differentially expressed levels of mRNA for ApoA-I translated into differential protein expression, Western blot analysis was performed on liver extracts from Mthfr^+/+ and Mthfr^−/− mice. Figure 2a shows reduced levels of the 27-kDa ApoA-I protein in Mthfr-deficient mice compared with wild-type mice. To quantify levels of ApoA-I, signal intensity was divided by the signal obtained from
Levels of ApoA-I protein and SD in liver of Mthfr+/− mice were 0.52 ± 0.12 of the value in Mthfr+/+ mice (4 pairs, P < 0.01). Western blot analysis was also performed on plasma from Mthfr+/+ and Mthfr+/− mice using albumin as internal control (Figure 2b); this analysis revealed that ApoA-I protein levels in plasma of Mthfr+/− mice were 0.62 ± 0.19 of the levels in Mthfr+/+ mice (7 pairs, P < 0.01). To ensure that the altered expression of ApoA-I related to the hyperhomocysteinemia rather than the disturbance in folate metabolism, we also performed Western blotting on plasma from mice with a heterozygous knockout of the cystathionine-β-synthase gene (Cbs+/−) (Figure 2c). These mice have hyperhomocysteinemia because of a defect in homocysteine transsulfuration instead of folate-dependent homocysteine remethylation. ApoA-I levels in Cbs+/− mice were 0.71 ± 0.16 of the levels in Cbs+/+ mice (2 pairs, 3 experiments, P < 0.01).

Correlation of Plasma Homocysteine With Levels of ApoA-I Protein and HDL Cholesterol in Males with Coronary Artery Disease

To determine whether our observation of reduced ApoA-I levels in hyperhomocysteinemic Mthfr-deficient and Cbs-deficient mice could be extrapolated to humans, we assessed ApoA-I protein levels in 60 plasma samples from a Montreal cohort of men with coronary artery disease, which we had previously examined for homocysteine and relevant nutrients. The mean tHcy value for this study was 10.5 μmol/L. We selected males with homocysteine levels above and below this mean for Western analysis, using albumin as an internal control (Figure 3). We observed lower levels of the ApoA-I protein in individuals with Hcy levels > 10.5 μmol/L (mean = 0.44, n = 29) compared with individuals with Hcy levels < 10.5 μmol/L (mean = 0.55, n = 31), P < 0.05. Furthermore, there was a negative correlation between levels of ApoA-I and plasma homocysteine with a significant r value of −0.33 (P < 0.01) (Figure 4a). We also observed a negative correlation between HDL cholesterol and plasma homocysteine in this group (r = −0.24); these findings had borderline significance (P = 0.06) (Figure 4b).

Figure 2. Representative Western blot for ApoA-I levels. Proteins from liver extracts (a) and plasma (b) of Mthfr+/− (lanes 1 and 3) and littermate Mthfr+/+ (lanes 2 and 4) mice. c, Proteins from plasma of Cbs+/− (lanes 1 and 3) and littermate Cbs+/+ (lanes 2 and 4) mice. Arrows indicate positions of ApoA-I (27 kDa) and loading controls β-actin (42 kDa) and albumin (68 kDa).

Figure 3. Representative Western blot for plasma ApoA-I levels from a male coronary artery disease population with Hcy levels > 10.5 μmol/L (lanes 1 to 4) and Hcy < 10.5 μmol/L (lanes 5 to 8). Arrows indicate positions of ApoA-I (27 kDa) and albumin (68 kDa, internal control).

Figure 4. Scatterplot analysis for a population of 60 plasma samples with a mean tHcy of 10.5 μmol/L. a, Negative correlation between ApoA-I and Hcy levels (r = −0.33, P < 0.01). b, Negative correlation between HDL cholesterol and Hcy levels (r = −0.24, P = 0.06).
Effect of Homocysteine on ApoA-I and PPARα Levels

To determine whether Hcy regulated ApoA-I through an effect on PPARα, HepG2 cells were treated with 0, 1, 5, and 10 mmol/L Hcy. A Hcy dose-dependent decrease in levels of both ApoA-I and PPARα was observed at 8 hours (Figure 5a); this decrease was evident as early as 4 hours and was sustained over 24 hours at 5 and 10 mmol/L Hcy (data not shown). Quantitation of ApoA-I and PPARα protein at 8 hours for 2 experiments showed a mean decrease of 10%, 40%, and 70% for ApoA-I and 30%, 40%, and 65% for PPARα, with increasing Hcy concentrations. These results are consistent with inhibition of PPARα by Hcy. Because PPARα is a regulator of ApoA-I, we suggest that the decrease in ApoA-I levels by Hcy is caused by PPARα downregulation.

We also compared effects of homocysteine and cysteine on ApoA-I expression in HepG2 cells. Western blotting (Figure 5b) revealed that ApoA-I expression was reduced by 47% with 5 mmol/L Hcy (a value similar to that in Figure 5a), whereas the same concentration of cysteine and the lower 1 mmol/L concentrations of both homocysteine and cysteine had little effect (14% or less of a decrease). These results suggest that the ApoA-I decrease is not attributable to a nonspecific effect of thiols.

Effect of Homocysteine on ApoA-I Promoter Activity

To determine whether the downregulation of ApoA-I by Hcy is caused by decreased activity of the ApoA-I promoter and if this decrease is mediated through PPARα-response element (PPRE), we constructed human apoA-I promoter-luciferase plasmids carrying wild-type PPRE (ApoAI-wt) or mutant PPRE (ApoAI-mut) (Figure 6a). HepG2 cells were transiently transfected with the constructs and after 6 hours, treated with Hcy (0, 1, or 5 mmol/L). The relative promoter activity with 0 homocysteine in cells transfected with ApoAI-mut was significantly lower than that in cells transfected with ApoAI-wt (43%, P<0.01, Figure 6b), indicating that the PPRE is a strong site driving the basal expression of the ApoA-I promoter. Similar results have been reported with an ApoA-II promoter construct in which a mutation in the PPRE (J-site) resulted in a strong decrease in baseline chloramphenicol acetyltransferase activity as well as a loss of inducibility by PPAR.31 When cells were transfected with ApoAI-wt plasmid, treatment with 1 and 5 mmol/L Hcy significantly decreased promoter activity to 89% (P<0.05) and 33% (P<0.01), respectively. With 5 mmol/L Hcy treatment, cells transfected with ApoAI-mut plasmid had decreased promoter activity (11%) compared with untreated cells (43%, P<0.01). Our results indicate that Hcy reduces ApoA-I promoter activity. However, because the mutant construct is also inhibited by Hcy, Hcy may be acting through a PPRE-independent mechanism.

Effect of PPARα on ApoA-I and MTHFR Expression

To confirm that PPARα affects ApoA-I synthesis in HepG2 cells, we cloned the human cDNA for PPARα into pCMV-Myc vector and performed transient transfection assays. In addition, we speculated that PPARα might also regulate MTHFR because sequences in the upstream region of the Mthfr promoter, identified through analyses using Visualization Tools for Alignment (VISTA software), contained a stretch of 5 nucleotides (GCACC) recognized as putative PPRE. This sequence was conserved and aligned between the mouse and human Mthfr promoters.

Western blotting (Figure 7a) showed that HepG2 cells transfected with PPARα had a 1.9-fold increase in ApoA-I protein compared with cells transfected with empty vector; total MTHFR protein also increased 1.6-fold (mean of 5 experiments, P<0.01). MTHFR has 2 protein isoforms, which differ in the length of their coding sequence and are generated by alternative splicing in the 5’ region.32 The 2 bands for MTHFR in Figure 7a consist of the shorter MTHFR isoform, which is posttranslationally modified by phosphorylation.33 To confirm the identity of the MTHFR isoforms in HepG2 cells, a protein extract from bacteria expressing only the shorter human MTHFR32 isoform was run (lane 1 in Figure 7b) and this band corresponded with the MTHFR isoform with increased mobility (nonphosphorylated) in HepG2 cells (lane 2 in Figure 7b). The increase in total MTHFR protein was observed for both the phosphorylated and nonphosphorylated forms. The longer MTHFR isoform was not observed in these cells. q-RT-PCR analysis con-
firmed the aforementioned increases, with an ~2-fold increase in mRNA levels for ApoA-I and a more modest 1.3-fold increase in MTHFR mRNA (data not shown).

**Discussion**

Although tHcy has become recognized as a risk factor for vascular disease, the biological mechanisms have not been completely elucidated. Several mechanisms have been proposed by which HHcy can contribute to endothelial dysfunction and atherosclerosis; these include oxidative stress, induction of proinflammatory factors, and endoplasmic reticulum stress.34 In this study, we identified a novel mechanism: reduced expression of the atheroprotective lipoprotein ApoA-I, the major protein component of HDL. We observed decreased mRNA for ApoA-I in liver and decreased ApoA-I protein in liver and plasma of Mthfr-deficient mice. These mice represent an excellent animal model for mild HHcy in humans. The mice have a 1.5- to 2-fold elevation in plasma homocysteine, lipid deposition in the valvular region of the aorta, and endothelial dysfunction; these findings are similar to those in humans with hyperhomocysteinemia with or without MTHFR deficiency. Other genetically engineered animal models have been used to study hyperhomocysteinemia, including mice with a complete knockout of cystathionine β-synthase (CBS−/−)35 or mice with knockouts in CBS and ApoE (CBS−/− and ApoE−/−).36 Other models of hyperhomocysteinemia have been generated by homocysteine infusions or by high dietary intake of homocysteine or methionine.37 Although these other models have been useful, the Mthfr−/− mice are a particularly attractive model because of their modest increase in homocysteine and the high natural occurrence of MTHFR deficiency in humans.

Our finding of reduced ApoA-I expression in CBS-deficient mice, as well as in MTHFR-deficient mice, suggests that it is the elevation in homocysteine, rather than the disturbance in folate metabolism, that regulates this apolipoprotein. Consistent with this argument are the effects of homocysteine on the ApoA-I promoter and in HepG2 cells, which were conducted in folate-replete medium.

The increase in mRNA for Cyp7A1 in liver of hyperhomocysteinemic mice is also consistent with enhanced atherogenesis. Cyp7A1 catalyzes 7α hydroxylation of cholesterol, the rate-limiting step in bile acid synthesis. Bile acids are transported from the liver to the intestine, where they facilitate cholesterol absorption by promoting the formation of micellar cholesterol.

Two key proteins involved in reverse cholesterol efflux, ApoA-I and Cyp7A1, were altered in expression in liver of Mthfr-deficient mice. Both proteins are regulated at the transcriptional level by the transcription factor PPARα,
which has been shown to increase ApoA-I expression\(^{30}\) and decrease Cyp7A1 expression.\(^{31}\) Our findings in HepG2 cells confirm the upregulation of human ApoA-I by PPAR\(\alpha\), because transfection with a PPAR\(\alpha\) construct increased ApoA-I levels and a mutated PPRE markedly reduced ApoA-I promoter activity. Homocysteine reduced the levels of PPAR\(\alpha\) and ApoA-I protein in HepG2 cells, suggesting that PPAR\(\alpha\) may be involved in homocysteine regulation of ApoA-I. Homocysteine also reduced ApoA-I promoter activity; however, a PPRE-independent mechanism could be involved because the ApoA-I promoter with a mutagenized PPRE was still subject to homocysteine inhibition. Although the homocysteine levels in the medium were higher than the nonphosphorylated isoform. Both isoforms are upregulated in response to PPAR\(\alpha\).

The regulation of MTHFR by PPAR\(\alpha\) is a novel finding that provides an additional interaction between homocysteine and lipid metabolism. A recent study has demonstrated that MTHFR activity can be regulated by phosphorylation; the phosphorylated enzyme is suggested to be less active than the nonphosphorylated enzyme.\(^{33}\) Our experiments showed increased expression of the phosphorylated and nonphosphorylated forms of MTHFR following transfection of HepG2 cells with PPAR\(\alpha\). These findings add more complexity to the regulation of MTHFR, which has 2 promoters, alternative splicing and extra coding sequences for the higher molecular weight isoform. Additional work is required to delineate the functional significance of this PPAR-dependent increase in MTHFR expression.

Our finding of homocysteine modulation of PPAR has also been suggested in studies of homocysteine-induced endothelial cell dysfunction. Homocysteine may antagonize the effects of PPAR\(\alpha\) and PPAR\(\gamma\) in endothelial cell remodeling.\(^{40}\) Interestingly, treatment of CBS-deficient mice with fibrates, which are known to induce PPAR\(\alpha\), have been reported to reverse the Hcy-mediated endothelial dysfunction in these mice.\(^{24}\)

The altered expression of ApoA-I in hyperhomocysteinemic mice was confirmed in a human cohort, a well-characterized group of males with coronary artery disease. In previous studies of this cohort, we showed that the MTHFR mutant genotype (677TT) was associated with increased plasma homocysteine only when folate status was low. Therefore, the association of homocysteine with reduced ApoA-I would only be expected in humans with the 677TT genotype when their folate status was low. We did not observe an association between the 677TT genotype and reduced ApoA-I levels in this study, because some of the males with this genotype had adequate folate and were therefore not hyperhomocysteinemic. Sampling of this population was performed before the introduction of folate fortification of food in Canada. It would be interesting to examine the association among folate, homocysteine, and apolipoproteins in North Americans after fortification.

In summary, we have demonstrated a significant negative relationship between levels of Hcy and ApoA-I in hyperhomocysteinemic males and in hyperhomocysteinemic Mthfr-deficient mice. Studies of large clinical populations are required to determine whether combined evaluation of both biomarkers is a better predictor of disease risk than either alone. In addition, it remains to be determined whether folate supplementation, which is known to reduce homocysteine levels, also modifies lipoprotein profiles.

Acknowledgments

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References


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**Table 1**

Changes in gene expression in 3 pairs of livers from 8 month-old *Mthfr*+− mice compared with *Mthfr*+/+ littermates.

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<td>hypermethylated in cancer 1 (Hic1)</td>
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GenBank accession numbers are shown on the left. Means of fold changes in gene expression in 3 experiments are shown on the right. Symbols ↑ and ↓ indicate increased and decreased gene expression in the livers of *Mthfr*+− mice with respect to *Mthfr*+/+ mice. The table shows only the genes with known function that were changed in expression in all 3 experiments.