Disturbed One-Carbon Metabolism Causing Adverse Reproductive Outcomes in Mice Is Associated with Altered Expression of Apolipoprotein AI and Inflammatory Mediators PPARα, Interferon-γ, and Interleukin-10

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Abstract

Low dietary choline or deficiency of methylenetetrahydrofolate reductase (Mthfr) leads to hyperhomocysteinemia (Hhcy) and adverse reproductive outcomes. Homocysteine reduces synthesis of ApoAI, the major lipoprotein in HDL-cholesterol; ApoAI is regulated by PPARα and has antiinflammatory properties. Our aim was to determine whether pregnancy complications due to genetic or nutritional deficiencies in 1-carbon metabolism could relate to dysregulation of ApoAI and inflammatory mediators. We fed pregnant mice, with or without a deficiency of Mthfr, control or choline-deficient (ChDD) diets for 10–12 wk and examined levels of ApoAI, PPARα, IFNγ, and IL-10. ApoAI mRNA was reduced in livers of Mthfr+/− mice and ApoAI protein was reduced due to Mthfr deficiency or choline deficiency in liver and plasma. Placental ApoAI protein was also reduced due to Mthfr genotype or choline-deficient diet and in developmentally delayed embryos. Reduced liver PPARα expression (mRNA and protein) was observed in ChDD-fed mice and was associated with increased methylation of a CpG dinucleotide in its promoter. Hepatic IFNγ increased due to genotype, and placental IFNγ was higher in Mthfr+/− ChDD-fed dams compared to Mthfr+/+ mice fed ChDD or Mthfr+/+ mice fed CD. IL-10 was reduced in livers of ChDD-fed mice. We propose that a deficiency of dietary choline or Mthfr leads to Hhcy and reduced expression of maternal ApoAI, with reduced ApoAI transfer to embryo. Disturbances in 1-carbon metabolism also reduce maternal PPARα expression, possibly through promoter hypermethylation, and increase IFNγ and decrease IL-10 levels. This disturbance of the T helper (Th1) (IFNγ:Th2 (IL-10) ratio and the increase in inflammatory mediators may contribute to pregnancy complications.

Introduction

Low intake of choline leads to Hhcy and adverse reproductive outcomes in rodents (1–3). Hhcy results from a disturbance in the metabolism of Hcy, because choline, through its metabolite betaine, can function as a methyl donor in folate-independent remethylation of Hcy to methionine (4). Folate-dependent remethylation of Hcy uses 5-methyl-THF as the methyl donor (4); this folate derivative is generated by MTHFR. We previously showed that reduced dietary intake of 1-carbon donors as well as genetic deficiency of Mthfr increases the incidence of pregnancy complications and disturbed embryonic development (1,5,6).

ApoAI, synthesized in liver and intestine, is the major protein component of HDL-cholesterol (7). It is protective against cardiovascular disease and exhibits antiinflammatory properties (8). Maternal HDL-cholesterol concentration and composition can affect the size of the fetus and sterol metabolism of the yolk sac and placenta in mice (9). During early and mid-pregnancy, maternal cholesterol contributes substantially to fetal cholesterol, whereas in late pregnancy, it is fetal cholesterol biosynthesis rather than transfer of maternal lipoproteins that meets the demands of the fetus (10).

In earlier work, we showed a link between ApoAI and Hcy metabolism (11,12) by demonstrating that Hhcy reduced ApoAI levels. Hhcy, a risk factor for cardiovascular disease (13), has also been associated with pregnancy complications (14,15). The most common genetic cause of mild Hhcy in humans is a polymorphism in MTHFR (677C→T) (16). We found a negative correlation between plasma Hcy and ApoAI in males with...
coronary artery disease (11). Mild Mthfr deficiency in mice (17), an animal model for the human 677 polymorphism, results in Hcy and decreased levels of ApoAI mRNA and protein in liver (11, 18). We suggested that Hcy may limit ApoAI synthesis in part through the nuclear receptor protein PPARα, because Hcy treatment of HepG2 cells resulted in decreased synthesis of PPARα and ApoAI (11). The anti-inflammatory properties of ApoAI (8) are partly mediated through down-regulation of neutrophil function (19) and suppression of the type I IFN receptor 2 (20). PPAR (subtypes α, β, and γ) also have anti-inflammatory properties; PPARα has been shown to suppress Th 1 immunity and promote Th2 immunity (21,22) and PPARγ is thought to promote placental growth through increased trophoblast differentiation and angiogenesis of fetal blood vessels (23). The Th1 and Th2 cytokines have opposite effects on human pregnancy and the inability of the mother to switch from Th1 to Th2 cytokine profiles at the fetal-maternal interface has been proposed as one of the primary causes of miscarriages, intrauterine growth retardation, and preeclampsia (24–26). IL-10 has anti-inflammatory properties through modulation of cytokine production by monocytes and neutrophils (27). A functional PPARE has been demonstrated in the promoter region of human IL-10 (28), suggesting a role for PPAR in the regulation of this cytokine.

We have been studying the impact of perturbed 1-carbon metabolism on adverse pregnancy outcomes in mice. The objective of the present study was to determine whether the increased pregnancy complications observed in mice with disturbed 1-carbon metabolism could be attributed to a disruption in regulation of ApoAI/PPARα or of other related immunomodulators.

Materials and Methods

Mice and diets. Animal experimentation followed the guidelines of the Canadian Council on Animal Care and was approved by the Montreal Children’s Hospital Animal Care Committee. Housing of mice, diets, timed matings, and collection of embryos and placentae were previously described (1). Briefly, Mthfr+/− and Mthfr−/− BALB/c female mice that had been backcrossed for at least 12 generations were placed after weaning on control diets (CD), which contained the recommended amount of nutrients for rodents (29) (choline bitartrate at 2.5 mg/kg diet and folic acid at 2 mg/kg diet), or choline-deficient diets (ChDD; with choline bitartrate at 0.3 mg/kg diet and folic acid at 2 mg/kg diet), or choline-deficient diets (ChDD; with choline bitartrate at 0.3 mg/kg diet and folic acid at 2 mg/kg diet), which were previously shown to achieve the desired increase in plasma Hcy levels (1). After 6 wk on diets, mice were mated with Mthfr−/− BALB/c male mice and the morning when a vaginal plug was present was considered as 0.5 dpc. Pregnant female mice were fed the same diets throughout pregnancy, and at 14.5 dpc, they were killed using a CO2 chamber after being maintained on the diets for a total of 10–12 wk. Maternal and placental tissues were collected and snap frozen. Evaluation of embryos and genotyping were performed as described (1,17).

Measurement of plasma Hcy. Blood was collected and centrifuged at 4500 × g for 7 min at 4°C to obtain plasma that was then frozen. tHcy was measured as before (1,6) using the A/C Portable Enzymatic Homocysteine Assay Kit and Reader following the manufacturer’s instructions (A/C Diagnostics).

Protein extraction and Western blotting. Protein extraction from liver and placenta was carried out using RIPA lysis buffer and Western blotting was performed as previously described (11) using antibodies against mouse ApoAI (Biodiens International), β-actin, IFNγ (Sigma-Aldrich), albumin (Bethyl Laboratories), PPARα (Santa Cruz Biotechnology), and IL-10 (Abbiotec). Signal detection was achieved with ECL-Plus Chemiluminescence System (GE Healthcare) and band intensities were quantified with Quantity One 4.6.6 software (Biorad) by 2 independent observers. Results from Western blotting are expressed relative to the mean value in Mthfr+/+ mice fed CD, which was standardized to a reference value of 1. Quantification of band intensities was performed by 2 observers, one of whom was unaware of the identity of the different groups. Because similar conclusions were derived from both observers, results from only one observer are presented.

Measurement of mRNA by qRT-PCR. RNA from liver and placenta was isolated using the RNeasy Mini kit (Qagen), CDNA was synthesized, and qRT-PCR performed as previously described (12). We used Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and primers that amplified mouse Apoai, Ppara, or Gapdh (served as a housekeeping gene) in a MX3000P QPCR system (Stratagene). Results from qRT-PCR are expressed relative to the mean value in Mthfr+/+ mice fed CD, which was standardized to a reference value of 1.

Quantitative DNA methylation analysis. Genomic DNA from liver was isolated by DNeasy blood and tissue kit (Qagen) and the methylation status of Ppara was evaluated by EpiTYPER (Sequenom platform, McGill University and Genome Quebec Innovation Center), which uses MALDI-TOF MS analysis with base-specific cleavage of single-stranded nucleic acids (30). Primers were designed in the CpG island of the promoter region in Ppara immediately upstream of the transcription start site; methylation of individual CpG nucleotides in this region has been reported to affect Ppara expression (31–33).

ELISA. Cytokine levels in maternal and embryonic tissues were assayed with the Mouse Th1/Th2 Ready-Set-Go! ELISA kit (eBioscience) following the manufacturer’s instructions. The kit measures levels of IFNγ and IL-10 in mouse plasma, serum, and culture supernatants. The procedure was slightly modified for use with tissue extracts. Plates were read in a PerkinElmer Wallac spectrophotometer (PerkinElmer Canada).

Statistical analysis. SPSS software version 11.0 was used for statistical analysis to compare means and to examine correlations. Two-factor ANOVA was performed to assess differences between diets and genotypes and diet × genotype interactions. Significant ANOVA results were followed by post hoc tests (Least significant differences for all figures except for figures 1A, 1C and 3C where Tukey’s test was used) to perform pairwise multiple comparisons. For some experiments, t tests were performed, where indicated, to assess differences within diet or genotype groups when ANOVA results were not significant. There was no accounting for unequal variances. Values represent mean ± SEM, n = number of mice per group. P < 0.05 was considered significant. Pearson’s correlation coefficients were calculated.

Results

Plasma Hcy and ApoAI levels in liver, plasma, and placenta of pregnant mice. Reproductive outcomes were reported earlier for the mice in these experiments (1). Mean plasma tHcy concentrations measured in this study (Fig. 1A) were consistent with previously reported values (5,6) and were elevated due to choline deficiency (P < 0.001) and Mthfr deficiency (P < 0.001), with an interaction between diet and genotype (P < 0.01).

Liver ApoAI protein and mRNA levels in Mthfr+/+ and Mthfr−/− dams fed CD or ChDD were measured by Western blot and qRT-PCR (Fig. 1B) and presented relative to β-actin and Gapdh, respectively. ChDD reduced ApoAI protein (P < 0.01) but not Apoai mRNA. Mthfr deficiency decreased hepatic ApoAI levels both at the protein (P < 0.05) and mRNA levels (P < 0.01). There was no significant diet × genotype interaction. The decrease in ApoAI protein at its site of synthesis in liver presumably led to the decrease in plasma ApoAI (Fig. 1C) in choline deficiency (P < 0.001) and in Mthfr deficiency (P < 0.05), with a dietary × genotype interaction (P < 0.01). We also observed a negative correlation between plasma tHcy and
Plasma tHcy (A), liver ApoAI protein and mRNA (B), and plasma ApoAI (C) levels in 3-mo-old pregnant Mthfr+/– and Mthfr−/− mice fed CD or ChDD. Values are means ± SEM, n = 5–7. Means without a common letter differ. In B, arrows on Western blots indicate positions of ApoAI and β-actin; ApoAI results are presented relative to β-actin. In C, ApoAI protein was measured by Western blot and presented relative to albumin. CD, control diet; CD−/−, Mthfr−/− mice fed control diet; CD++/−, Mthfr−/− mice fed control diet; ChDD−/−, Mthfr−/− mice fed choline-deficient diet; ChDD−/+, Mthfr−/− mice fed choline-deficient diet; ChDD−/−, Mthfr−/− mice fed choline-deficient diet; ChDD, choline-deficient diet.

PPARα expression and promoter methylation in pregnant mice. To identify mechanisms that could explain the reduced levels of maternal liver ApoAI, we examined levels of the transcription factor PPARα, which is a known regulator of ApoAI. Western blotting showed that ChDD decreased the levels of hepatic PPARα (Fig. 3A) at the protein (P < 0.05) and mRNA levels (P < 0.01); there was no effect due to Mthfr genotype and no diet × genotype interaction. In addition, there was a positive correlation between liver PPARα protein and ApoAI protein levels (r = 0.55; P < 0.05) (Fig. 3B). We were not able to detect PPARα mRNA or protein in placental extracts from E14.5 dpc embryos by qRT-PCR or Western blot. Although it is unclear whether PPARα is expressed in placenta at this gestational time point (34), it is possible that our methods may not have been sufficiently sensitive to detect limited amounts of placental PPARα at E14.5 dpc.

Because Ppara expression can be regulated by DNA methylation in rats (31–33), we performed methylation analysis of CpG dinucleotides in the Ppara promoter region using the EpiTYPER platform to assess whether choline deficiency reduced Ppara mRNA expression through an increase in DNA methylation. The region of interest spanned over 450 bp; among the 30 potential CpG dinucleotides analyzed in this region, a single CpG (UCSC assembly mm9, chr15:85566055) was found to be ≈10–20% methylated in livers of mice fed CD (Fig. 3C); all other sites in this 450-bp region had virtually no methylation (≈5%). Ppara promoter methylation was higher at this CpG site (≈30% methylation) in livers of mice fed ChDD compared to mice fed CD (P < 0.01) and there was a significant (50%) decrease in promoter methylation in CD-fed Mthfr−/− mice compared to CD-fed Mthfr+/− mice (P < 0.05, t test). There was no diet × genotype interaction.

Levels of IFNγ and IL-10 in liver and placenta of pregnant mice. Because we found reduced PPARα levels during choline
deficiency and because PPARα can affect the type of immune response (Th1 or Th2), we assessed the levels of major cytokines in the Th1 (IFNγ) and Th2 (IL-10) responses in liver and placenta. In maternal livers, Mthfr deficiency increased IFNγ levels, as measured by ELISA (Fig. 4A) (P < 0.01), and a negative correlation was observed between maternal hepatic ApoAI and IFNγ protein levels (Fig. 4B) (r = −0.67; P < 0.01); no significant dietary effect or diet × genotype interaction was observed for IFNγ. Levels of maternal liver IL-10 were measured by Western blot, which detected the dimer and monomer forms of this cytokine (Fig. 4C). Values for IL-10 dimer, monomer, and the ratio of dimer:monomer for each group were separately examined and presented relative to the value in CD-fed Mthfr+/+ mice. Choline deficiency tended to reduce the levels of the IL-10 dimer (Fig. 4D) (P = 0.09), which is reported to be the biologically active form of the cytokine (35,36). However, both choline and Mthfr deficiency increased levels of the biologically inactive IL-10 monomer (Fig. 4E) (P < 0.01 for diet, genotype, diet × genotype interaction, ANOVA), suggesting that Hhcy maintains IL-10 in its inactive form, possibly preventing the conversion of monomer to active dimer. The presence of 2 bands for the dimer in the CD-fed mice, but not in ChDD-fed mice, may reflect different amounts of glycosylation, which does not affect the biological activity of IL-10 (37,38). Analysis of the ratio of the active IL-10 dimer:inactive monomer revealed a reduction of this ratio in choline deficiency (Fig. 4F) (P < 0.01). Of additional interest was a positive correlation between maternal liver PPARα and the IL-10 dimer (r = 0.64; P < 0.05) and a borderline positive correlation between maternal liver PPARα and the IL-10 dimer:monomer ratio (r = 0.66; P = 0.08) (data not shown). Baseline levels of maternal IFNγ and IL-10 in plasma of these mice were too low to be measured by conventional methods.

Placental protein extracts from Mthfr+/+ and Mthfr−/− embryos of Mthfr+/+ and Mthfr−/− dams, respectively, fed CD or ChDD were assayed by ELISA (Fig. 5A) and revealed elevated IFNγ in Mthfr−/− embryos of mothers fed ChDD compared to Mthfr+/+ embryos of mothers fed CD (P < 0.01, t test) or compared to Mthfr+/+ embryos in the ChDD group (P < 0.05, t test). The placental IFNγ levels in Mthfr+/+ and Mthfr−/− mice fed CD, as well as Mthfr+/+ mice fed ChDD, were at the basal detection level of the ELISA kit. Therefore, to confirm the ELISA findings in placenta, we also assessed IFNγ levels in placenta by Western blot; a representative blot is shown in Figure 5B, where one distinct band of ~52-kDa molecular weight was detected. Although the predicted molecular weight of murine IFNγ is reported to be ~15 kDa, higher molecular weight proteins ranging from 40 to 80 kDa have also been reported (39). There was no evidence of a 15-kDa band in our blots, even at higher exposure. Quantification of Western blots (Fig. 5B) confirmed the findings seen with ELISA, i.e., increased placental IFNγ levels in Mthfr−/− embryos of mothers fed ChDD compared to Mthfr+/+ embryos of mothers fed CD (P < 0.05, t test) or compared to Mthfr+/+ embryos in the ChDD group (P < 0.01, t test). Moreover, as shown for maternal liver (Fig. 4B), there was a significant negative correlation between ApoAI and IL-10 proteins in placenta from Mthfr−/− embryos of dams fed ChDD (r = −0.77; P < 0.05) (data not shown).

**Discussion**

Nutritional or genetic deficiencies in 1-carbon metabolism result in Hhcy, which has been associated with a variety of pregnancy complications, including early reproductive loss, intrauterine growth retardation, preeclampsia, and birth defects (15). In previous work, we have shown that hyperhomocysteinemic mice with disturbances in 1-carbon metabolism have increased resorptions, placental abnormalities, embryonic delayed development, and growth retardation (1,5,6). The present study was undertaken to determine whether the increased pregnancy complications observed in mice with disturbed 1-carbon metabolism, due to genetic or nutritional deficiencies, could be attributed at least in part to fluctuations in maternal or fetal PPARα, ApoAI, and related cytokines. We therefore studied Mthfr-deficient mice, as a model for the common 677C→T
MTHFR polymorphism, that were fed diets with reduced levels of choline.

Apoa1 mRNA levels were reduced in livers of pregnant mice with Mthfr deficiency. This finding is consistent with our earlier observations in which nonpregnant, Mthfr-deficient mice exhibited lower ApoAI mRNA and protein in liver (11) and Hcy decreased the synthesis of ApoAI in HepG2 cells (12). We did not observe ApoAI mRNA changes in placental tissue in this study due to either Mthfr or choline deficiency, because the main sites of ApoAI synthesis are liver and intestine. The decreased synthesis of maternal hepatic ApoAI resulted in lower levels of ApoAI protein in liver, plasma, and placenta, as seen in Mthfr-deficient dams fed ChDD. The reduced levels in placenta are presumably due to decreased transfer of circulating ApoAI from mother to embryo. The negative correlation between maternal plasma Hcy and maternal plasma ApoAI protein is due to the reduced ApoAI in the developing heart. The regulation of ApoAI synthesis by PPARα was of particular interest in this study, because PPARα has a major role in the regulation of placental growth and in inflammation (42,43). Maternal administration of PPARγ agonists during mid-pregnancy increases the rate of spontaneous fetal mortality by one-half (44). PPARα signals anti-inflammatory properties by increasing the production of Th2 cytokines (IL-10 and IL-4) and decreasing production of Th1 cytokines (IFN-γ and IL-2) (22). Inflammatory processes may alter the balance predominantly toward a Th1 response, which initiates a cascade of inflammatory cytokine production involved in spontaneous abortion, preterm delivery, and preeclampsia (21,45); in particular, IFN-γ has been shown to be elevated in preterm deliveries (46) and placental IL-10 deficiency has been observed in women with preeclampsia (47). We observed ~50% decreased Ppara mRNA and ~25% decreased protein in maternal liver due to a deficiency in dietary choline. One regulatory mechanism that could account for reduced Ppara expression is altered DNA methylation, because dietary manipulation in pregnant rats has been shown to alter the methylation of specific cytosines in the Ppara promoter region and affect gene expression (31–33). Analysis of CpG dinucleotides in the promoter region of Ppara revealed ~50% increased methylation at a particular cytosine residue in livers of ChDD-fed mice; this observation correlates with the reduced hepatic expression of Ppara mRNA and protein, as mentioned above.

Mthfr deficiency resulted in reduced methylation of the Ppara promoter. Pregnant mice fed CD, but no changes were observed for PPARα expression; this may be due to the fact that PPARα is already highly expressed in liver. We previously observed global DNA hypomethylation in Mthfr-deficient mice in several tissues (17), because MTHFR is required for synthesis of methionine and the global methyl donor S-adenosyl-methionine; the hypomethylation in Ppara may therefore relate to the generalized hypomethylation. Dnmt1 methylation may also explain some of the changes in Ppara methylation, because choline deficiency during rat embryonic development has been shown to affect DNA methylation by hypomethylation of CpG in the

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of mice with Mthfr deficiency and in placentae of Mthfr-deficient mice with reduced intake of choline. The changes in IFNγ expression may also relate to the hypermethylation and reduced expression of PPARα. In addition, a significant negative correlation was observed between IFNγ and ApoAI both in maternal liver and in placentae, especially under low dietary choline. IL-10 is also known to inhibit IFNγ production by lymphocytes (56). These correlations between ApoAI, IFNγ, and IL-10 are consistent with a common regulator such as PPARα, thus leading us to suggest that reduced PPARα expression may result in decreased ApoAI and increased IFNγ in maternal tissues and placenta. It is also possible that disturbed 1-carbon metabolism or the subsequent Hcy increases IFNγ levels independently of its effect on PPARα through effects on oxidative stress and inflammation (57–59). In a mouse model of nonalcoholic steatohepatitis, C57Bl mice were fed a methionine- and choline-deficient diet that resulted in decreased HDL levels and increased inflammation and lipoperoxidation in liver (60).

We have observed nutritional (low dietary choline) and genetic (Mthfr deficiency) influences on the levels of some inflammatory mediators during pregnancy, which may contribute to pregnancy complications. The combination of ChDD with Mthfr deficiency had the greatest impact on plasma Hcy, ApoAI, IL-10, and IFNγ in all tissues examined. To determine whether it is the maternal or fetal increase in proinflammatory molecules and/or the decrease in antiinflammatory markers that cross the placenta requires more in-depth analysis.

In summary, we suggest that some of the adverse reproductive outcomes observed in mice with a disruption of 1-carbon metabolism could be attributed at least in part to decreased maternal PPARα and ApoAI synthesis, which results in reduced placental transport of ApoAI to the fetus. The perturbations in PPARα also create an imbalance in the Th1:Th2 cytokine ratio through increased maternal and fetal IFNγ and reduced maternal IL-10. Changes in the levels of these inflammatory mediators in maternal and placental tissue could also contribute to pregnancy complications.

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**Literature Cited**


promoter of Dnmt1, leading to DNMT1 overexpression and a subsequent increase in both global and gene-specific hypermethylation (48). Changes in histone methylation, which can result in gene activation or gene silencing (49,50), have been described during choline deficiency (51,52) and could be another epigenetic modification regulating transcriptional control of PPARα. Alternatively, other CpG sites upstream of the Ppara promoter, possibly in “Cpg island shores” (53), may undergo methylation changes that could affect PPARα expression. The distinct patterns of methylation of the Ppara promoter in Mthfr and choline deficiency require additional study.

In light of the presence of a PPRE in the promoter region of Apoa1, the hypermethylation and reduced transcription of Ppara in choline deficiency may translate into lower levels of ApoAI. The role of PPARα in the Th2 host response and the putative PPRE in the antiinflammatory cytokine IL-10 are also of interest in this regard, because choline deficiency also decreased maternal hepatic levels of IL-10. PPARα levels positively correlated with ApoAI and IL-10 levels in maternal liver, consistent with regulation of both molecules by this transcription factor. PPARα also regulates the immune response through a decrease in Th1 cytokines (54,55). We found increased levels of the proinflammatory cytokine IFNγ in livers...


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