CHAPTER 6

Riboflavin and Methylenetetrahydrofolate Reductase

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Abstract

The flavoenzyme methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl group donor in the conversion of homocysteine to methionine. In rats, experimental riboflavin deficiency is associated with low MTHFR activity and reduced levels of 5-methyltetrahydrofolate. In humans, reduced enzyme activity caused by the commonly occurring 677C→T substitution of the *MTHFR* gene is associated with elevated plasma homocysteine. The mutant enzyme has lower affinity for its flavin cofactor than the wild-type enzyme, and recent studies show that plasma homocysteine is inversely related to riboflavin in subjects with the T-allele. This indicates that the metabolic effect of the 677C→T polymorphism is related to riboflavin status, which may have implications for future studies on the relationship between this polymorphism and various clinical and biochemical endpoints.

Introduction

Riboflavin is a water-soluble vitamin, which serves as the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).[1] FMN is formed by the phosphorylation of riboflavin, and FAD is formed in a subsequent ATP-dependent reaction as most of the FMN is adenylated.[1] FMN and FAD are cofactors for more than 150 reduction-oxidation enzymes, some of which are involved in the metabolism of folate, vitamin B6 and cobalamin [1,2] (Fig. 1).

The majority of flavoenzymes, including methylenetetrahydrofolate reductase (MTHFR), are FAD-dependent.[1,3] Mammalian MTHFR is a cytosolic homodimer, and each subunit contains a catalytic N-terminal domain as well as a regulatory C-terminal domain,[4] which binds the allosteric inhibitor S-adenosylmethionine (AdoMet).[3,4] The enzyme uses NADPH as a cofactor in addition to FAD, and catalyzes the transformation of 5, 10–methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-methylTHF). The reaction is irreversible in vivo and is the only source of 5-methylTHF, which serves as the methyl donor for the cobalamin-dependent conversion of homocysteine to methionine.[5] In most tissues, this provides the sole pathway for homocysteine remethylation. Concentrations of homocysteine in tissues and blood also depend upon its degradation through vitamin B6-dependent transsulfuration in the liver and kidneys.[5]

Thermolabile MTHFR associated with lower catalytic activity was reported approximately 15 years ago.[6] Later, this thermolability was shown to be caused by a 677C→T transition in the *MTHFR* gene, resulting in an alanine to valine substitution in the enzyme.[7] MTHFR in lymphocytes from subjects with the TT genotype has approximately 30% of the catalytic activity of the wild-type, while the CT genotype has 65% of the catalytic activity.[7] The frequency of

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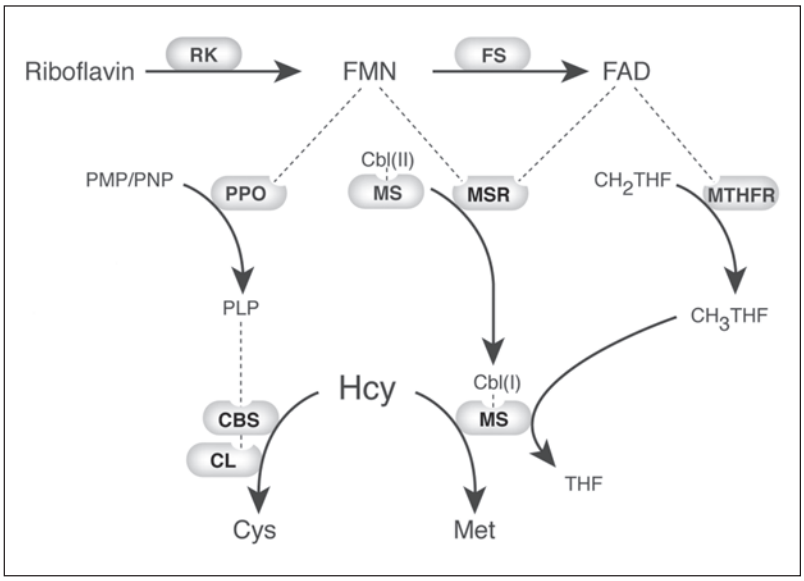

Figure 1. Riboflavin is linked to the metabolism of B-vitamins involved in homocysteine remethylation and transsulfuration. Riboflavin is transformed to FMN and FAD by the action of riboflavin kinase (EC 2.7.1.26) and FAD synthase (EC 2.7.7.2). FMN and FAD are cofactors for vitamin B6, cobalamin and folate metabolizing enzymes. Vitamin B6 and cobalamin serve as cofactors for enzymes involved in homocysteine metabolism, while folate serves as a one-carbon donor for the remethylation of homocysteine. Abbreviations: Cbl(I)= cob(I)alamin; Cbl(II)= cob(II)alamin; CBS= cystathionine β-synthase; CH_2-THF= 5, 10-methylenetetrahydrofolate; CH_3-THF= 5-methyltetrahydrofolate; CL= cystathionine lyase; Cys= cysteine; FS= FAD synthase; Met= methionine; MS= methionine synthase; MSR= methionine synthase reductase; MTHFR= 5, 10-methylenetetrahydrofolate reductase; PLP= pyridoxal-5′-phosphate; PMP= pyridoxamine-5′-phosphate; PNP= pyridoxine-5′-phosphate; PPO= pyridoxine-5′-phosphate oxidase; RK= riboflavin kinase; TH= tetrahydrofolate.

the T-allele is about 0.3-0.4 in several populations of Asian and European descent, but is much lower in sub-Saharan Africa and in some other areas.[8]

Guenther et al studied the biochemical properties of MTHFR in an E. coli model.[9] They found that the enzyme variant homologous to the human Ala222Val substitution had lower binding affinity for FAD than the wild-type, and that folate stabilized the enzyme and increased FAD affinity.[9] Purified human MTHFR was later shown to have similar biochemical properties.[4] Folate and AdoMet stabilized the enzyme, particularly the mutant, and protected against flavin loss. The stabilizing effect of FAD was studied with respect to loss of enzyme activity after heating and after enzyme dilution, and in both cases the flavin cofactor was found to have a protective effect.[4]

Animal Studies

In 1968, Narisawa et al investigated the relationship between riboflavin status and MTHFR activity, and found lower enzyme activity in livers of riboflavin-deficient rats than in controls.[10] Bates and Fuller confirmed these findings by demonstrating a dose-dependent relationship between riboflavin status and MTHFR activity.[11] They also reported that MTHFR was particularly sensitive to riboflavin deficiency, and that enzyme activity decreased relatively more than intracellular concentrations of FAD when riboflavin was scarce. Low MTHFR activity in riboflavin deficiency is associated with altered distribution of liver folates, and 5-methylTHF is reduced relative to other folate forms.[10-12] Redistribution of folates may explain why the

urinary excretion of formiminoglutamic acid after histidine loading is reduced in riboflavin-deficient rats compared to controls,[10,12] and possibly why homocysteine is elevated in the skin of riboflavin-deficient rats.[13]

MTHFR activity has also been investigated in hypothyroid rats.[14-16] Hypothyroidism is associated with low intracellular FMN and FAD, which has been explained by low activity of riboflavin kinase, the enzyme catalysing the conversion of riboflavin to FMN[17,18] (Fig. 1). As with alimentary riboflavin deficiency, MTHFR activity is low,[15] and intracellular levels of 5-methylTHF are reduced relative to other folates.[14-16] Histidine oxidation is increased,[14-16] both at high and low intakes of methionine.[14,15] Hypothyroidism is also associated with higher levels of AdoMet, which may inhibit MTHFR activity.[16]

Human Studies

Assessment of Riboflavin Status

Glutathione reductase is a FAD-dependent enzyme, which is sensitive to riboflavin deficiency.[19] This probably explains why the erythrocyte glutathione reductase activation coefficient (EGRAC), which is the ratio between in vitro enzyme activity determined with and without the addition of FAD, is useful for the assessment of riboflavin status.[20,21] High levels of EGRAC indicate low FAD saturation of the apoenzyme and biochemical riboflavin deficiency. The method has been used as an indicator of riboflavin status in several clinical studies.[21]

In some human studies, concentrations of plasma riboflavin have been used for the assessment of riboflavin status.[22,23] This parameter appears to be a more sensitive indicator of riboflavin status than plasma concentrations of flavin cofactors.[24] In severe riboflavin deficiency, plasma concentrations of FAD are probably lowered as well.[24,25]

Blood Levels of tHcy and Folate in MTHFR Deficiency

The role of MTHFR in homocysteine remethylation is illustrated by the finding that both mild MTHFR deficiency, which is observed in subjects with the 677C→T transition, and severe MTHFR deficiency are associated with elevated concentrations of plasma tHcy.[7,8] High plasma tHcy in subjects with the TT genotype is usually observed only under conditions of impaired folate status,[26] which probably reflects the role of folate as a substrate and as a genotype-dependent regulator of enzyme stability. Moreover, the TT genotype is frequently associated with low levels of plasma folate.[27] 5-methylTHF is the predominant folate species in plasma,[27] and low folate is probably related to impaired synthesis of 5-methylTHF. Published data on erythrocyte folate may appear contradictory, and increased[27] or decreased[28] concentrations have been reported in subjects with the TT genotype compared to the CT and CC genotypes. Such apparent inconsistencies may be method dependent and reflect the ability of different assays to detect various cellular folate species.[29] This idea is supported by the finding of formylated tetrahydrofolates in erythrocytes from subjects with the TT genotype, whereas CC cells contain only 5-methylTHF.[30]

Riboflavin Intake and Plasma tHcy

The relationship between dietary riboflavin and plasma concentrations of tHcy has been studied in a few cross-sectional studies.[31-33] In individuals from the Framingham Offspring cohort (n = 1960), plasma tHcy was 1.0 μmol/l higher in the lowest compared to the highest quintile of riboflavin intake in a multivariate model adjusted for intakes of folate, cobalamin, vitamin B6 and other possible determinants of tHcy.[31] Samples were collected prior to the implementation of mandatory folic acid fortification in 1998, and individuals who were regular users of B-vitamin supplements were excluded from the analysis.[31] A strong inverse relationship between riboflavin intake and plasma tHcy was reported in another American study, but the data were not adjusted for folate or other B-vitamins.[32] Dietary intakes of folate, riboflavin, vitamin B6 and cobalamin were inversely related to plasma tHcy in 2435 men and women from a Dutch population-based cohort,[33] but only folate remained associated with

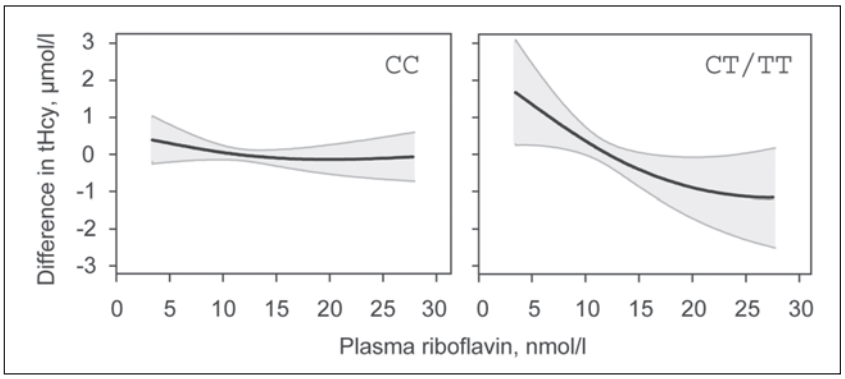

Figure 2. Dose-response curves for the relationship between plasma riboflavin and plasma homocysteine according to *MTHFR* 677C→T genotype. Solid lines indicate estimated dose-response curves, and shaded areas represent 95% confidence intervals. The curves are obtained by additive Gaussian regression analysis, and the model is adjusted for sex, age, serum folate, serum cobalamin, and serum creatinine. The figure is modified from reference 22 with permission.

tHcy in a multivariate model, adjusted for B-vitamins and other determinants of plasma tHcy. Individuals who used B-vitamin supplements were excluded from the study. None of the above studies investigated the possible effect of *MTHFR* 677C→T genotype on the riboflavin-tHcy relationship.[31,32]

Riboflavin and the MTHFR *677C→T Polymorphism As Determinants of tHcy*

An inverse relationship between plasma concentrations of riboflavin and tHcy was reported by Hustad et al in a study of 423 Norwegian blood donors[22] (Fig. 2). Plasma tHcy was 1.4 µmol/l higher in the lowest compared to the highest riboflavin quartile in a multiple regression model adjusted for folate and other determinants of tHcy. The riboflavin-tHcy relationship was modified by the *MTHFR* 677C→T polymorphism and was essentially confined to subjects with the T allele (Fig. 2). The riboflavin-tHcy relationship was not significantly modified by levels of serum folate.

Jacques et al studied 450 subjects from the Framingham Offspring cohort, selected according to the *MTHFR* 677C→T polymorphism and equally distributed between the CC, CT and TT genotypes.[23] They found an inverse association between plasma concentrations of riboflavin and plasma tHcy, but only in subjects with the TT genotype and plasma folate below the median (12.5 nmol/l). In this group, tHcy was 2.9 µmol/l higher in the lowest compared to the highest riboflavin tertile after adjustment for sex, age and folate. There was no relationship between concentrations of flavin cofactors and tHcy.[23]

Thus, both the American and the Norwegian studies[22,23] demonstrated an association between riboflavin and tHcy, which was dependent on the *MTHFR* 677C→T polymorphism. In the Framingham Offspring cohort, the riboflavin-tHcy relationship was weaker, however. In addition, plasma tHcy was less strongly associated with the *MTHFR* genotype, and concentrations of serum folate were independent of genotype.[22,23] Thus, the polymorphism apparently had less metabolic effects in the American than in the Norwegian study.

This disparity might be explained by different intakes of vitamins or nutrients, which modulate MTHFR activity. Riboflavin intake was probably higher in the Americans, because grain products have been riboflavin fortified in the USA since the 1940s[23,34] and levels of fortification have been rising over the years.[34] Although samples were collected prior to the implementation of mandatory folic acid fortification in the USA, many breakfast cereals were fortified at the time of the study,[23,34] and folate status may have been better in the Americans. The idea

that vitamin intake differs between the two populations is further supported by the finding of an overall correlation between concentrations of riboflavin and folate in the American study (Spearman correlation coefficient = 0.31, P <0.001),[23] but not in the Norwegian study.[22] Such a correlation might be related to common dietary sources for these vitamins.

In a recent study by McNulty et al of 286 healthy individuals from Northern Ireland, EGRAC was used to determine riboflavin status.[35] The authors demonstrated a significant inverse association between riboflavin status and plasma concentrations of tHcy. As in previous studies, this relationship was modified by *MTHFR* genotype, and in the lowest tertile of riboflavin status, mean tHcy was 18.09 μmol/l in the TT group as compared to 10.15 μmol/l in the CT and 8.32 μmol/l in the CC groups. When riboflavin status was higher, tHcy was independent of genotype. The genotype-tHcy relationship was also dependent on folate, and was only observed in subjects with concentrations of erythrocyte folate below the median.[35] The authors did not assess the combined effects of riboflavin, folate and *MTHFR* genotype on tHcy in a multivariate model, but they found no correlation between riboflavin and erythrocyte folate,[35] which makes serious confounding from folate unlikely.

The relationship between plasma tHcy and riboflavin status has also been investigated in end-stage renal disease.[36] In a study of 54 nonvitamin supplemented patients with a mean age of 54 years who were maintained on peritoneal dialysis, mean plasma tHcy was 33.0 μmol/l. Ten patients had EGRAC equal to or greater than 1.52, indicating riboflavin deficiency.[36] The authors found a positive association between EGRAC and tHcy, which is consistent with high tHcy when riboflavin status is low. Riboflavin was significantly related to plasma tHcy in multivariate models, which included folate and other B-vitamins. The riboflavin-tHcy relationship was not studied in relation to the *MTHFR* 677C→T polymorphism, because of the relatively low number of patients.[36]

Riboflavin Intervention Studies

The homocysteine lowering effect of 15 days of riboflavin (10 mg/d; n = 10) or vitamin B6 (20 mg/d; n = 10) supplementation was investigated in a small study of riboflavin and vitamin B6-deficient Indian women.[37] Mean tHcy was 12.1 and 14.7 μmol/l in the riboflavin and B6 groups, respectively, and tHcy decreased only in the B6 group.[37] There was no placebo group, and no data on *MTHFR* 677C→T genotype.

Another riboflavin intervention study was published by McKinley et al.[38] In the first phase of this study, 46 subjects with suboptimal riboflavin status (EGRAC ≥ 1.20) received low-dose riboflavin (1.6 mg/d; n = 23) or placebo (n = 23) for 12 weeks. In the second phase of the study, participants originally on placebo received 400 μg/d of folic acid for 6 weeks followed by a combination of folic acid and riboflavin for 12 weeks. Folic acid supplementation lowered tHcy, but no effect of riboflavin was observed in either phase, and apparently riboflavin status was not a determinant of plasma tHcy in this study. A possible reason is that only five subjects had the TT genotype. In addition, riboflavin status was only modestly impaired, and no subject had EGRAC higher than 1.40.[38]

Implications

MTHFR may be sensitive to riboflavin status,[11] particularly in subjects with the 677C→T substitution of the *MTHFR* gene. In subjects with the TT genotype, higher riboflavin intake could be necessary for the formation of adequate amounts of 5-methyl-THF involved in homocysteine remethylation. Although the TT genotype comprises only around 10% of the population in many countries,[8] it is more prevalent in subjects with hyperhomocysteinemia. In a Norwegian population-based study of men and women aged 40 – 67 years, 73% of individuals with plasma tHcy equal to or higher than 40 μmol/l had the TT genotype, compared to 10% of the controls.[39] In men from Northern Ireland, the TT genotype occurred in 48, 35, and 23% of the top 5, 10, and 20% of individuals ranked by plasma tHcy levels.[40] This indicates that the *MTHFR* 677C→T polymorphism may be important for the development of moder-

ate hyperhomocysteinemia, and an effect of the enzyme variant associated with the TT genotype might be partly attributed to riboflavin.

Much of the interest in the *MTHFR* 677C→T polymorphism stems from its association with moderate hyperhomocysteinemia, which is a risk factor for occlusive arterial disease, venous thrombosis, neural tube defects and pregnancy complications.[5,8] It is still not clear whether elevated tHcy is the cause of these conditions or if it is mainly a surrogate marker. In several studies, the *MTHFR* 677C→T polymorphism itself has been shown to modify the risk of certain diseases,[8] including cardiovascular disease,[41,42] neural tube defects[27] and colon cancer,[43,44] while other studies show no such relationship.[8] Inconsistent reports on the *MTHFR* 677C→T polymorphism and disease risk could be explained through effect modification by nutritional factors. This has been demonstrated for folate,[42,44] whereas the role of riboflavin has received less attention.[45]

Further research is warranted to investigate the importance of riboflavin as a regulator of MTHFR activity, particularly with respect to its interaction with folate and its relationship to various clinical endpoints.

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