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Defective remethylation of homocysteine is related to decreased synthesis of coenzymes B2 in thyroidectomized rats

A. Ayav[1,2], **J. M. Alberto**[1], **F. Barbe**[1], **L. Brunaud**[1,2], **P. Gerard**[1], **M. Merten**[1], and **J. L. Gueant**[1]

[1] Laboratory of Cellular and Molecular Pathology in Nutrition, INSERM-0014, Medical Faculty, University of Nancy I, Vandoeuvre lès Nancy, France
[2] Department of Digestive Surgery, University Hospital Brabois, Vandoeuvre les Nancy, France

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Summary. We investigated the influence of hypothyroidism on homocysteine metabolism in rats, focusing on a hypothetical deficient synthesis of FAD by riboflavin kinases. Animals were allocated in control group (n = 7), thyroidectomized rats (n = 6), rats with diet deficient in vitamin B2, B9, B12, choline and methionine (n = 7), thyroidectomized rats with deficient diet (n = 9). Homocysteine was decreased in operated rats (2.6 ± 1.01 vs. $4.05 \pm 1.0\,\mu$mol/L, P = 0.02) and increased in deficient diet rats (29.56 ± 4.52 vs. $4.05 \pm 1.0\,\mu$mol/L, P = 0.001), when compared to control group. Erythrocyte-Glutathione-Reductase-Activation-Coefficient (index of FAD deficiency) was increased in thyroidectomized or deficient diet rats (P = 0.004 for both). Methylenetetrahydrofolate-reductase and methionine-synthase activities were decreased in thyroidectomized rats but not in those subjected to deficient diet. Cystathionine-β-synthase was increased only in operated rats. Taken together, these results showed a defective re-methylation in surgical hypothyroidism, which was due in part to a defective synthesis of vitamin B2 coenzymes. This defective pathway was overcompensated by the increased Cystathionine-β-synthase activity.

Keywords: Thyroidectomy – Homocysteine – Vitamin B2 – MTHFR – MTR – CBS

Introduction

Homocysteine is an amino acid of the one carbon metabolism, which is associated with the risk for cardiovascular diseases, neural tube defects, neurodegenerative disorders, and digestive carcinogenesis (McCully, 1969; Steegers-Theunissen et al., 1991; Mikol et al., 1983; Zapisek et al., 1992). Its plasma level is influenced by pathological events such as deficiency in folate, deficiency in vitamin B12, renal failure and thyroid dysfunction (Nebredo et al., 1998; Barbé et al., 1999; Nair et al., 1994; Guldener et al., 1999). Methionine synthase (MTR) and betaine-homocysteine-methyl-transferase (BHMT) play a key role in homocysteine metabolism, as they catalyze the synthesis of methionine by re-methylating homocysteine (Fig. 1). BHMT dependant remethylation needs betaine as a co-substrate. S-adenosyl methionine (SAM), the substrate of methylation of DNA, proteins and lipids, is also an inhibitor of methylenetetrahydrofolate reductase (MTHFR) and an activator of Cystathionine β-synthase (CBS), the key enzyme of the trans-sulfuration pathway of homocysteine. MTR dependant remethylation needs the presence of methyltetrahydrofolate as a co-substrate and B12 (methylcobalamin) as a co-factor of MTR (Banerjee and Matthews, 1990; Bestor, 1998; Razin and Riggs, 1980). MTR reductase keeps MTR in active form by reducing the cobalt of vitamin B12 during the catalytic cycle while methyltetrahydrofolate is synthesized from methylenetetrahydrofolate by MTHFR. Both MTHFR and MTR reductase need vitamin B2 as a coenzyme.

We have previously found a decreased synthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in thyroid dysfunction related to malnutrition (Capo-Chichi et al., 2000). This may result from the influence of thyroid hormones on the expression of flavokinases and FAD synthetase, the enzymes that catalyse the synthesis of FMN and FAD from riboflavin (vitamin B2) respectively (Rivlin, 1970).

We hypothesize therefore that a failure of coenzyme B2 synthesis by flavokinase may affect homocysteine re-methylation pathway in hypothyroidism (Fig. 1). The present study was undertaken to investigate this hypothesis in rats subjected to thyroidectomy and/or a methyl-deficient diet.

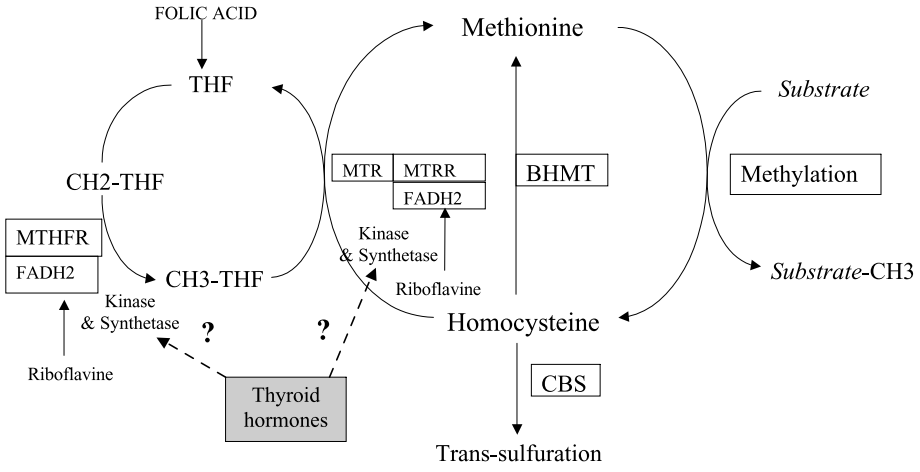


Fig. 1. Hypothetical influence of thyroid hormones on the Vitamin B2 coenzymes (FADH2) dependant enzyme of the one carbon metabolism. *THF*, Tetrahydrofoloate; *CH2THF*, methylene tetrahydrofolate; *CH3THF*, methyltetrahydrofolate; *MTHFR*, methylene tetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; *BHMT*, betaine homocysteine methyl transferase; *CBS*, cystathionine β synthase; *FADH2*, flavin adenine dinucleotide

Materials and methods

Animals

Twenty-nine male Fisher F344 rats were purchased. They were 1 per cage in plastic cages throughout the study at a room temperature of $23 \pm 3°C$, under a 12 h dark cycle. Free access to tap was allowed. All rats were fed with normal diet for four week before starting the experiment. After this environmental adaptation period, all animals underwent blood sample collection. Animals were then allocated in 4 study groups:

- Group 1 (control group): non operated rats submitted to normal diet (n = 7),
- Group 2: thyroidectomized rats submitted to normal diet (n = 6),
- Group 3: non operated rats submitted to methyl-deficient diet (n = 7),
- Group 4: thyroidectomized rats submitted to methyl-deficient diet (n = 9).

In the current study, normal diet was defined as standard food (Maintenance diet M20, Scientific animal food an engineering, Villemoisson-sur-Orge, France); deficient diet was free vitamins B2 and B12, folate, methionine and choline (Special Diet Service, Saint-Gratien, France). Thyroidectomy was performed by a surgical approach: animals were anaesthetized by intraperitoneal injection of ketamin 25 mg/kg and a vertical cervicotomy was performed. The thyroid gland was exposed and carefully dissected under binocular glasses (×4) (Zeiss; USA) with a special attention for parathyroid glands and recurrent nerves. Blood samples were collected from anaesthetized animals by arteria caudalis mediana puncture.

Rats were sacrificed 8 weeks after the inclusion in the groups. Animals were anaesthetized by intraperitoneal injection of ketamin 25 mg/kg, and following a midline abdominal incision and sternotomy, blood samples were collected from heart puncture. Each blood samples were collected on heparin and then centrifuged for 5 min at 3500 rpm. Aliquots of plasma were stored frozen at $-80°C$ until analysis. Liver were rapidly removed, freeze-clamped in liquid nitrogen, and stored at $-80°C$ until further used.

Body weight and blood samples were checked the first and the last day of the study. Blood samples were used to determine plasma homocysteine, blood TSH, vitamin B12 and folate levels at the beginning and at the end of the study. The activities of the enzymes involved in methionine metabolism and vitamin B2 status were assessed only at the end of the study.

Assays of TSH, vitamin B2, B12, folate, homocysteine

Blood TSH level was determined by competitive radio immunoassay (Immunotech; Beckman Coulter Company). Vitamin B2 status has been assessed by measuring the Erythrocyte Glutathione Reductase Activation Coefficient (EGRAC) which is the ratio between enzyme activity determined with and without the addition of the cofactor FAD (Glatzle, 1970; Glatzle, 1973; Becker, 1991). Cobalamin and folate levels were determined by radio-dilution isotope assay (SimulTRAC-SNB; ICN Pharmaceuticals- USA). Plasma homocysteine level was determined by fluorescent polarization immunoassay (FPIA) (IMX system Abbott; Norway).

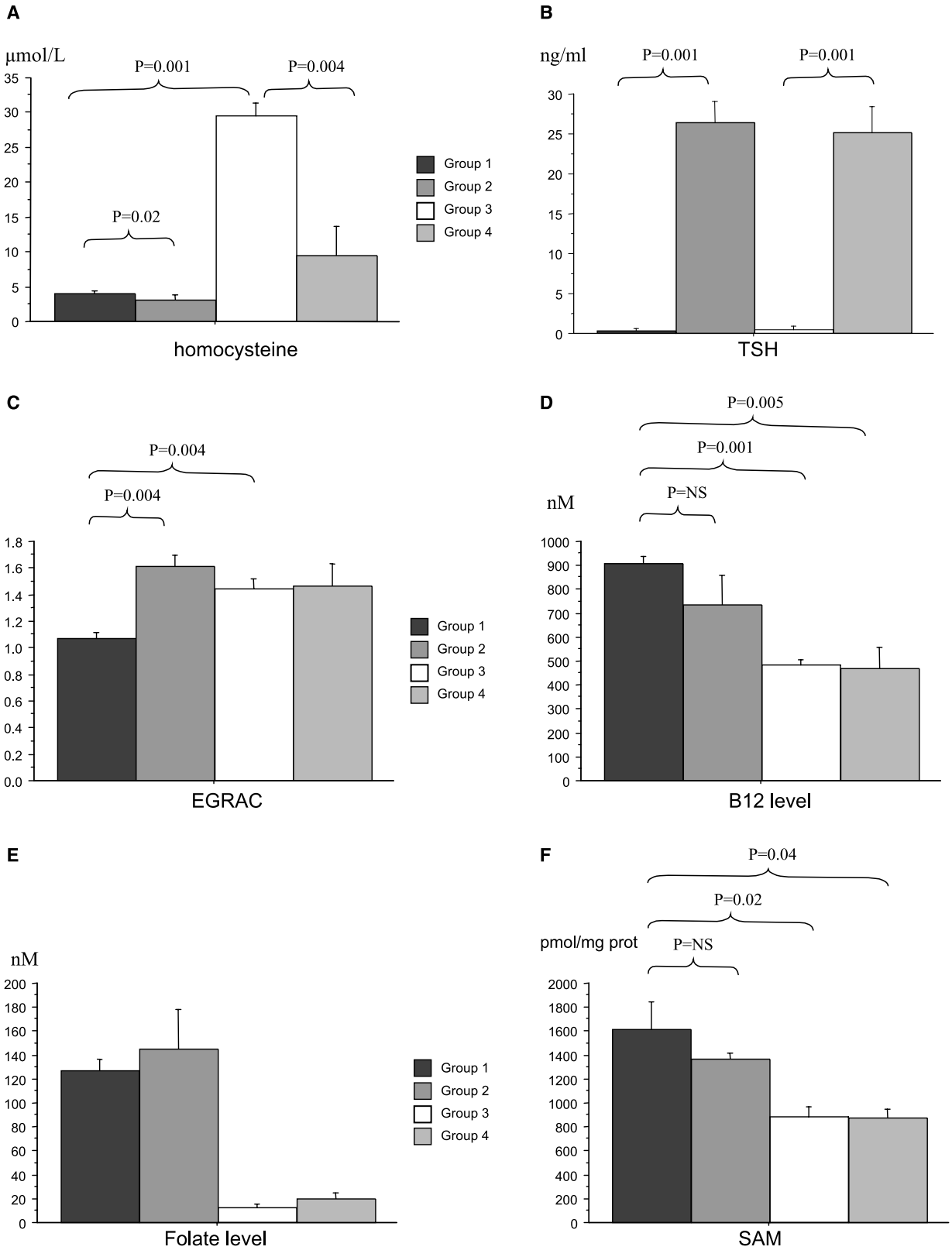
Activities of enzymes involved in methionine metabolism

Methionine synthase (MTR): the method was adapted from Chen *et al.* (Chen, 1995). Proteins were incubated in a buffered solution consisting of 100 mM KH_2PO_4 at pH 7.2, 25 mM DTT, 25 mM ascorbate, 0.5 mM SAM, 50 μM OH-cobalamin, 5 mM D,L-HCY (freshly prepared) and 250 μM [$^{14}CH_3$]methyl-tetrahydrofolate ($\sim$11,000 cpm/nmol). The reaction was stopped by adding cold water; after centrifugation, the supernatant was laid on a cationic gel (quaternary ammonium, AG1 × 8, Cl^- form, Bio-Rad). After elution, [$^{14}CH_3$]methionine was measured with PicoFluor-40 (Packard Biosciences).

Betaine-homocysteine methyltransferase (BHMT) enzymatic activity was measured as described by Garrow (Garrow, 1996). *Methylene Tetrahydrofolate Reductase (MTHFR)* activity was measured as reported by Kutzbach et al. (1971).

Cystathionine β-synthase (CBS): the method was adapted from Taoka et al. (1998). Proteins were incubated for 30 min at 37°C in a reactive solution

Fig. 2. Plasma homocysteine level and related determinants in rats subjected to thyroidectomy and/or deficient diet. Data are expressed as mean ± SD. **A** Homocysteine plasma level; **B** TSH plasma level; **C** Erythrocyte Glutathione Reductase Activation Correlation test (EGRAC); **D** Plasma vitamin B12; **E** Plasma folate; **F** S-adenosylmethionine (SAM) in liver extracts



Group 1: Control group
Group 2: Thyroidectomized rats. normal diet
Group 3: Non operated rats. deficient diet
Group 4: Thyroidectomized rats. deficient diet

containing 0.1 M Tris (pH 8.5), 1 mM cystathionine, 5 mM D,L-serine (Sigma), 1 mM pyridoxal 5′-phosphate, 0.25 μCi (^{14}C) serine (Amersham Biosciences, Saclay, France), and 15 mM homocysteine (Sigma). After washing with 20 mM maleic acid (pH 1.9) and 0.2 M LiCl, radiolabeled cystathionine was eluted with 50 mM acetic acid (pH 4.8), 0.8 M LiCl into Dowex 50WX2-200 gel (Sigma), and then quantified by means of a liquid scintillation analyser (Packard Biosciences, Meriden, CT, USA).

S-Adenosylmethionine (SAM): the method was adapted from Luippold et al. (1999) and based on a reverse phase liquid chromatography technique using a linear acetonitrile gradient. Proteins were precipitated with 0.2 N $HClO_4$, centrifuged, and the supernatant was filtered through 0.45 μm before injection on the column (Lichrospher®, 100 RP-C18, 5 μm, 250 × 4 mm I.D). The mobile phase was applied at a flow rate of 0.75 mL/min and consisted in 50 mM sodium phosphate (pH 3.2), 10 mM heptan sulfonate and acetonitrile (10 to 20% from 0 to 20 minutes). Amounts of SAM was quantified by using an UV detector (254 nm).

Statistical analysis

The analysis was performed using StatView (SAS Institute Inc.; Berkeley, USA). All values were expressed as mean ± standard deviation. Mann-Whitney-U test was used to evaluate differences between continuous variables. Correlations were evaluated using correlation matrix and stepwise multiple regression analysis. Statistical significance was considered for P values < 0.05.

Results

Homocysteine level was highly increased in rats of group 3 (non operated rats submitted to deficient diet) when compared to control group (29.56 ± 4.52 vs. 4.05 ± 1.0 μmol/L, $P = 0.001$). Inversely, homocysteine level was significantly decreased in rats of group 2 (thyroidectomized rats submitted to normal diet) when compared to control group (2.6 ± 1.01 vs. 4.05 ± 1.0 μmol/L, $P = 0.02$) (Fig. 2A). Moreover, homocysteine level was dramatically decreased in rats subjected to both thyroidectomy and deficient diet (group 4) when comparing rats submitted to rats subjected only to deficient diet (group 3) (9.55 ± 9.0 vs. 29.56 ± 4.52 μmol/L, $P = 0.004$). These findings showed that thyroidectomy decreased homocysteine level whatever the nutritional status in methyl precursors was.

TSH level at the end of the study was significantly increased in groups of thyroidectomized rats (groups 2 and 4) when compared to non operated rats (groups 1 and 3) (Fig. 2B), indicating the efficiency of the surgical procedure for producing hypothyroidism. Thyroid and/or diet status had no significant influence on the evolution of plasma creatinine and urea levels between levels at the inclusion day into the study and levels at the end of the study. These changes were equivalent in all 4 groups.

The EGRAC value was significantly increased in thyroidectomized rats (1.61 ± 0.21 vs. 1.07 ± 0.12, $P = 0.004$), with the same order of magnitude than that observed in rats subjected to a diet deficient in vitamins B

Table 1. Values of enzymes involved in homocysteine metabolism

	Non operated normal diet Group 1 (n = 7)	Thyroidectomized normal diet Group 2 (n = 6)	P-value (Group 2 vs. 1)	Non operated deficient diet Group 3 (n = 7)	P-value (Group 3 vs. 1)	Thyroidectomized deficient diet Group 4 (n = 9)	P-value (Group 4 vs. 1)
MTHFR (nmol/h/mg)	5.12 (±2.94)	2.37 (±0.28)	0.01	2.27 (±0.35)	0.01	2.15 (±0.47)	0.01
MTR (nmol/h/mg)	3.26 (±0.31)	2.43 (±0.63)	0.01	2.99 (±0.47)	NS	2.20 (±0.60)	0.007
CBS (nmol/h/mg)	194 (±60)	355 (±95)	0.01	262 (±79)	NS	215 (±73)	NS
BHMT (nmol/h/mg)	14.6 (±7.0)	14.8 (±4.3)	NS	10.7 (±2.0)	NS	9.5 (±2.9)	NS

MTHFR, Methylene tetra hydro folate reductase
MTR, Methionine synthase
CBS, Cystathionine β synthase
BHMT, Betaine homocysteine methyl transferase

Table 2. Correlation between TSH and metabolic and nutritional markers of one carbon metabolism

	Correlation coefficient	Z	95% CI	P-value
TSH vs				
Homocysteine	−0.469	−2.331	−0.734, −0.081	0.01
EGRAC	0.523	2.661	0.152, 0.765	0.007
MTR	−0.603	−3.197	−0.809, −0.264	0.001
Homocysteine vs				
B9	−0.670	−3.718	−0.845, −0.366	0.0002
B12	−0.524	−2.664	−0.765, −0.152	0.007
SAM	−0.567	−2.804	−0.798, −0.191	0.005
EGRAC vs				
TSH	0.523	2.661	0.152, 0.765	0.007
MTHFR	−0.393	−1.905	−0.688, 0.012	0.05

EGRAC, Erythrocyte glutathione reductase activation correlation
MTHFR, Methylene tetra hydro folate reductase
MTR, Methionine synthase
SAM, S-adenosylmethionine

(1.44 ± 0.19 vs. 1.07 ± 0.12, $P = 0.004$) (Fig. 2C). The increased EGRAC value indicated a decreasing on vitamin B2 status. As expected, the levels of serum folate and B12 were lower in rats subjected to deficient diet, and was not influenced by thyroidectomy (Fig. 2D and E).

The SAM level was not influenced by thyroidectomy and was decreased by 2-fold in rats subjected to deficient diet (groups 3 and 4) confirming, at a metabolic level, its efficacy (Fig. 2F).

Regarding enzymes involved in re-methylation pathway in thyroidectomized rats (group 2), MTHFR and MTR activities were decreased when compared the control group ($P = 0.01$ for both). Conversely, CBS activity, was highly increased ($P = 0.01$). On the other hand, regarding the same enzymes in deficient diet rats (group 3), only MTHFR activity was significantly decreased compared to control group ($P = 0.01$). Rats subjected to both thyroidectomy and deficient diet (group 4) exhibited a decreased MTHFR and MTR activities ($P = 0.01$ and 0.007 respectively), as observed in those undergoing thyroidectomy alone (Table 1).

In multiple regression analysis, blood TSH level was significantly correlated with MTR, EGRAC and homocysteine (Table 2). The association of TSH level with EGRAC indicated therefore that thyroid hormones are involved in regulating the synthesis of riboflavin coenzymes. The weak association of MTHFR activity with EGRAC ($P = 0.05$) illustrated the already known role of coenzyme B2 in MTHFR activity. The absence of significant correlation between TSH and MTHFR could be due to the limited number of rats ($p = 0.1065$).

Discussion

This study was designed to investigate the influence of surgical hypothyroidism on homocysteine metabolism in rats, concomitantly with the already known nutritional determinants of homocysteine. We focused our interest in the status on FAD and its consequences on the re-methylation pathway. The dramatically increasing of blood TSH level in operated rats showed the efficiency of this surgical model to produce hypothyroidism. We preferred this surgical approach to a ''medical thyroidectomy'' with propylthiouracil or with radioactive iodine (Rivlin, 1970) in order to eliminate a possible effect of this treatment on homocysteine metabolism.

Contrarily to what has been observed in human hypothyroidism (Ingenbleek et al., 1986; Nebredo et al., 1998; Barbé et al., 1999), hypothyroid rats exhibited a tendency toward a decrease of plasma homocysteine level, in agreement to what has been previously reported by Jacobs et al. (2000). In a previous work, we reported an increase of homocysteine level around 20 μmol/L after 2 months in rats subjected to diet deficient in folate, vitamin B12, methionine and choline but not in vitamin B2 (Brunaud et al., 2003). The current deficient diet included the removal of vitamin B2. Under these conditions a higher concentration of homocysteine was observed (29 μmol/L), illustrating the importance of vitamin B2 coenzymes in homocysteine metabolism in rats. We observed an increase of EGRAC values in thyroidectomized rats in the same order of magnitude than in rats subjected to a diet deficient in vitamin B2. In addition,

EGRAC was significantly correlated to TSH ($r = 0.523$, 95% CI: 0.152–0.765, $P = 0.007$). These 2 findings showed therefore a decrease synthesis of FAD by riboflavine kinases as previously reported in hypothyroidism (Rivlin and Langdon, 1966; Cimono et al., 1987; Lee et al., 1985). We believe that the low MTHFR activity observed in thyroidectomized rats could be therefore explained by an impaired synthesis of coenzymes B2 riboflavine by riboflavine kinase (Glatzle, 1973). Two experimental findings supported this hypothesis: 1) a low MTHFR activity was reported in hypothyroid rats, which was in the same order of magnitude than that recorded in the rats deficient in vitamins B (2.37 ± 0.28 and 2.27 ± 0.35 nmol/h/mg, respectively); and 2) MTHFR activity was significantly correlated with EGRAC ($r = -0.393$, 95% CI: -0.688–0.012, $P = 0.05$).

Hypothyroid rats exhibited a significant decreasing of MTR activity. This decreased activity observed in rats with hypothyroidism was not depending on vitamin B12 since the level of this vitamin was not different to that reported in the control group. Even though no correlation was found with EGRAC, MTR activity is depending indirectly on FAD, which is the co-enzyme of MTR reductase. Presently, MTR activity was determined in presence of DTT, a reductase chemical agent that does not allow to evaluate the influence of MTR reductase and FAD.

Taken together, our results suggested that hypothyroidism produced a defective re-methylation of homocysteine, with a drop of the activity of the two key enzymes, MTHFR and MTR that could be related in part to a decreased synthesis of FAD. Because of a defective re-methylation pathway, we would have expected hyperhomocysteinemia in hypothyroid rats, as described in hypothyroid humans (Ingenbleek et al., 1986; Nebredo et al., 1998; Barbé et al., 1999). Furthermore, this defective re-methylation was not compensated by BHMT suppletive pathway since the activity of this enzyme in the liver was not influenced by hypothyroidism. In addition, homocysteine metabolism of operated rats was not affected by renal dysfunction as creatinine and urea levels remained unchanged in rats submitted tothyroidectomy and/or deficient diet. In fact, the tendency to hypohomocysteinemia of the thyroidectomized rats can be explained by an increased CBS activity, involved in the transsulfuration pathway. This increased activity was not related to an allosteric activation by SAM, as the level of this effector was not affected by thyroidectomy. This means probably that the increased activity of CBS, which has been already observed by others (Heinonen, 1975; Jacobs et al., 2000) played a predominant role on the homocysteine homeostasis compared to the defective re-methylation pathway, at least in hypothyroid rats.

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Authors' address: Prof. Jean-Louis Gueant, Laboratory of cellular and molecular pathology, INSERM-0014, Medical Faculty, University of Nancy I, avenue de la forêt de Haye, 54500, Vandoeuvre lès Nancy, France,

Fax: +33.3.83.15.35.91, E-mail: jl.gueant@chu-nancy.fr