Effects of supplementary folic acid and vitamin B₁₂ on hepatic metabolism of dairy cows according to methionine supply¹

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ABSTRACT

The present experiment was undertaken to study the interactions between dietary supplements of rumen-protected methionine (RPM) and intramuscular injections of folic acid and vitamin B₁₂, given from 3 wk before calving to 16 wk of lactation, on hepatic metabolism of lactating dairy cows. Sixty multiparous Holstein cows were assigned to 10 blocks of 6 cows each according to their previous milk production. Within each block, 3 cows were fed a diet calculated to supply Met as 1.83% of metabolizable protein, whereas the 3 other cows were fed the same diet supplemented with 18 g of RPM calculated to provide Met as 2.23% of metabolizable protein. Within each level of Met, the cows received no vitamin supplement or weekly intramuscular injections of 160 mg of folic acid alone or combined with 10 mg of vitamin B₁₂. Liver biopsies were taken at 2, 4, 8, and 16 wk of lactation. Liver concentrations of folates and vitamin B₁₂ were increased by their respective supplements but this response to vitamin supplements was altered by methionine supply. Concentrations of total lipids and triglycerides increased in livers of cows fed RPM, whereas concentrations of cholesterol ester, cholesterol, diglycerides, phosphatidylethanolamine, and phosphatidylcholine were not affected. Folic acid, alone or combined with vitamin B₁₂, tended to increase the ratio of phosphatidylcholine to phosphatidylethanolamine. Gene expression of 5,10-methylene-tetrahydrofolate reductase, microsomal transfer protein, and phosphatidylethanolamine methyltransferase were higher in liver of cows fed RPM supplements. The relative mRNA abundance of 5,10-methylene-tetrahydrofolate reductase and methylmalonyl-CoA mutase were

increased by the combined injections of folic acid and vitamin B₁₂, whereas those of methionine synthase and methionine synthase reductase were not affected by treatments. These results suggest that increasing supply of methyl groups, as preformed labile methyl groups or through methylneogenesis, affected the methylation cycle but had a limited effect on dairy cow performance. The observed effects of the combined supplement of folic acid and vitamin B₁₂ on lactational performance of dairy cows probably result from an improvement of energy metabolism during early lactation.

Key words: dairy cow, folic acid, vitamin B₁₂, rumen-protected methionine

INTRODUCTION

The roles of folic acid, vitamin B₁₂, and Met are closely interrelated in the methylation cycle (Figure 1). Folic acid deficiency reduces the activity of the methylation cycle by decreasing methylneogenesis. Vitamin B₁₂ is the coenzyme of methionine synthase, the enzyme essential for the transfer of a methyl group from the 5-methyl-tetrahydrofolate to homocysteine (**Hcy**) for regeneration of Met. This amino acid is the precursor of S-adenosylmethionine (**SAM**), the major donor of methyl groups. A vitamin B₁₂ deficiency blocks the transfer of a methyl group from 5-methyl-tetrahydrofolate leading to a secondary folate deficiency by interfering with folate utilization in cells (Scott, 1999). Supply of Met, because of its role as donor of preformed labile methyl groups, affects the needs for methylneogenesis (Bailey and Gregory, 1999) and consequently, for these 2 vitamins.

Besides their roles in the methylation cycle, folic acid is involved in purine and pyrimidine synthesis, and consequently, DNA synthesis and cell division; vitamin B₁₂ plays a major role in the entry of propionate in the Krebs cycle and gluconeogenesis, through the vitamin B₁₂-dependent enzyme methylmalonyl-CoA mutase (**MUT**; McDowell, 2000); and Met has an important role in protein synthesis (Brosnan et al., 2007).

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Figure 1. Methylation cycle and simplified vitamin B₁₂-dependent pathways. Key enzymes: 1 = methionine adenosyltransferase; 2 = phosphatidylethanolamine methyltransferase; 3 = glycine N-methyltransferase; 4 = S-adenosylhomocysteine hydrolase; 5 = methionine synthase (MS); 6 = methionine synthase reductase; 7 = 5,10-methylenetetrahydrofolate reductase; 8 = betaine homocysteine methyltransferase; 9 = cystathionine β -synthase; 10 = cystathionine β -lyase; 11 = propionyl-CoA carboxylase; 12 = methylmalonyl-CoA mutase. 5-CH₃-THF = 5-methyl-tetrahydrofolate; 5,10-CH₂-THF = 5,10-methylene-tetrahydrofolate; THF = tetrahydrofolate; PS = phosphatidylserine; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine.

Recent studies demonstrated that, under some circumstances, supplementary folic acid and vitamin B₁₂ improved lactational performance of dairy cows without altering DMI (Girard and Matte, 2005; Graulet et al., 2007). However, given the intricate metabolic pathways dependent on these 2 vitamins, it is not clear if these effects were mediated through changes in DNA synthesis, methylneogenesis, or gluconeogenesis. Girard and Matte (2005) observed that administration of vitamin B₁₂ to dairy cows fed a diet supplemented with folic acid and rumen-protected Met (**RPM**) increased lactational performance and blood hemoglobin concentrations but decreased plasma concentrations of methylmalonic acid. These results suggest that low vitamin B₁₂ supply interfered with the vitamin B₁₂-dependent enzyme MUT, as well as with folate metabolism because folic acid deficiency, through its role in DNA synthesis, affects

hematopoiesis (Bills et al., 1992). However, Graulet et al. (2007) reported that supplementary folic acid, given alone or in combination with vitamin B₁₂, increased milk and milk protein yields without affecting DMI, suggesting that these responses were not dependent on vitamin B₁₂ supply. However, in this last study, when vitamin B₁₂ was given with folic acid, changes in concentrations of plasma glucose and hepatic lipids suggested an improvement in metabolic efficiency. These last effects were possibly related to an improvement of gluconeogenesis efficiency because an increased affinity of MUT for its cofactor, vitamin B₁₂, in hepatocytes of cows simultaneously fed folic acid and vitamin B₁₂ supplements was also observed.

Given the close interaction of folic acid and vitamin B₁₂ in the methylation cycle and glucose metabolism, these 2 vitamins should play an important role in the

regulation of these metabolic pathways in lactating dairy cows. In addition, because the liver plays a key role in the coordination of nutrient fluxes to support lactation, it was hypothesized that supplements of these 2 vitamins would improve metabolic efficiency at the hepatic level. In an attempt to further delineate the modes of action of folic acid and vitamin B₁₂ on hepatic metabolism, different metabolites and expression of genes closely related to the roles described above of these 3 nutrients have been studied.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

Sixty multiparous Holstein cows from the herd at the Agriculture and Agri-Food Canada Research Centre (Sherbrooke, Quebec, Canada) were assigned to 10 blocks of 6 cows each according to their previous milk production; treatments were tested according to a 2 × 3 factorial arrangement. Within each block, 3 cows were fed the basal diet calculated to supply Met as 1.83% of MP (**M**−), whereas the 3 other cows were fed the same diet supplemented daily with RPM [**M**+: 9 and 18 g of Mepron-85 (Degussa AG, Hanau, Germany) per day, pre- and postcalving] to bring the estimated Met supply to 2.23% of MP in the lactation diet (NRC, 2001). Within each level of Met (**M**+ or **M**−), the cows received no vitamin supplement (**B**₉−**B**₁₂−) or weekly intramuscular injections of 160 mg of folic acid alone (**B**₉+**B**₁₂−; pteroylmonoglutamic acid, ICN Biochemicals Inc., Cleveland, OH) or in combination with 10 mg of vitamin B₁₂ (**B**₉+**B**₁₂+; cyanocobalamin, 5,000 µg/mL, Vetoquinol, Lavaltrie, Québec, Canada).

The experimental period began 3 wk before the expected date of calving and lasted until 16 wk after calving. Cow management and diet composition are described in Preynat et al. (2009a).

Sampling Procedures

Liver biopsies were performed at 13.9 ± 1.3, 27.8 ± 1.4, 55.8 ± 1.4, and 112.1 ± 1.8 d after calving under local anesthesia and ultrasound guidance to minimize the hemorrhagic risks as described by Graulet et al. (2007). The procedure was approved by the Institutional Committee on Animal Care of the Sherbrooke Research Centre in accordance with the guidelines of the Canadian Council of Animal Care (1993). Liver tissue was immediately blotted to remove blood, frozen into liquid nitrogen, and stored at −80°C until used.

Laboratory Analysis

Folates and Vitamin B₁₂. Concentrations of folates and vitamin B₁₂ in liver were determined in duplicate

by radioassay using a commercial kit designed for human plasma [Quantaphase FolatesII/vitamin B₁₂, Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada] as described by Graulet et al. (2007). The interassay coefficients of variation were 4.4 and 2.0% for folates and vitamin B₁₂, respectively.

Lipids Fractions in Liver. Cholesterol ester, triglycerides, diglycerides, and cholesterol in hepatic tissue were extracted according to the method described by Folch et al. (1957) and quantified using a HPLC method adapted from Homan and Anderson (1998). The dry lipid extracts were dissolved in 5 mL of isooctan/tetrahydrofuran (9/1) (Sigma, St. Louis, MO) and stored at −20°C until HPLC analysis. The HPLC system components were from Kontron Instruments (Zürich, Switzerland) and consisted of a P325 pump, a 360 autosampler, and vacuum degasser 200 (PerkinElmer, Courtaboeuf, France). All components were controlled by the Kromasystem 2000 software. A 150- × 4.6-mm column containing silica 5 µm (Sunfire Silica 5 µm, Waters, Guyancourt, France) and equipped with a 0.2-µm column prefilter (Waters) was heated to 40°C in a column thermostat 482 (Kontron Instruments). Lipids were detected with a SEDEX 85 evaporative light scattering detector (Sedere S.A., Alfortville, France), equipped with nitrogen generator (Parker Balston N2-04, Parker Hannifin, La Chaussee Saint Victor, France), set to 50% of maximum gain, a drift tube temperature of 45°C, and a nitrogen pressure of 3.5 bar.

Extraction, separation, and quantification of total lipids, phosphatidylethanolamine (**PE**), and phosphatidylcholine (**PC**) were performed according to the methods of Christie (1985) and Becart et al. (1990).

RNA Extraction and cDNA Synthesis. Total RNA was extracted from liver tissue using TRIzol reagent (Gibco BRL, Burlington, Ontario, Canada) according to the manufacturer's instructions. Total RNA was dissolved in water and quantified spectrophotometrically at 260 nm, and an aliquot was run on 1% agarose gel to verify its integrity. Total RNA was reverse transcribed to cDNA in a PTC-200 Peltier Programmable Thermal Cycler (MJ Research, Foster City, CA). Five micrograms of total RNA was treated with 3 units of DNase I (amplification grade; Gibco BRL) to remove contaminating genomic DNA. First-strand cDNA was synthesized using a superscript II preamplification system (Gibco BRL) and 500 ng of oligo(dT)₁₂₋₁₈ as primer (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) in 50 µL of total reaction volume.

Cloning and Sequencing of 5,10-Methylene-Tetrahydrofolate Reductase, Betaine Homocysteine Methyltransferase, and Methionine Synthase Reductase Bovine Genes. To determine the bovine-specific sequence of 5,10-methylene-tetrahy-

Table 1. Primer sequences and optimal conditions for PCR of bovine selected genes

Primer name ¹	Primer sequence (5'-3') ²	Primer position (nt)	GenBank accession no.	Length (bp)	Annealing temperature (°C)
Cloning					
BHMT-F	CAGTTTCGYCAGCTTCATCGRGAG	218–240	AY854632	489	50
BHMT-R	GGCAGTTTCACRCCRAYRATGGAKG	706–683			
MTHFR-F	TGGARACCATCCTGCAYATGA	553–573	AY854631	612	50
MTHFR-R	CGCGGTTGAGGGTGTAGAAAG	1164–1145			
MTRR-F	GTKCCAATTTCAAARGCMRTT	831–851	DQ084520	516	50
MTRR-R	TTTAGGAAGRTGYTCSAGCAG	1346–1326			
RACE³					
BHMT-5'	ATGCTTTCAAGGCTTCAACTGCC	583–561	AY854632	583	
BHMT-3'	CATGCAGACCTTCACCTTCTATG	264–286		1,602	
MS-5'	TTCTGTCTGGAGAGAGATGTCC	433–411	DQ084519	433	
MS-3'	CGGCACAAGCTGAGTGAAGAAGA	148–170		3,685	
MTHFR-5'	TTGCGGATGGCAGCATCATTGT	1075–1054	AY854631	1,075	
MTHFR-3'	CTGGAGGTGCCACAGCAGATCA	1008–1029		1,593	
MTRR-5'	CTGCTGCACAGCTCCTGCAGCC	1225–1204	DQ084520	1,225	
MTRR-3'	GTGACGATTAAGGCAGACACGAG	1032–1054		1,121	
Real-time PCR					
ACTB-F	GCGTGGCTACAGCTTCACC	624–642	AY141970	54	60
ACTB-R	TTGATGTCACGGACGATTTTC	677–658			
BHMT-F	TGTCAAGAAGGGCATCCTAGAAC	78–100	AY854632	70	58
BHMT-R	AAAGACAAACCCTCCGTCTCC	147–127			
CYCLO-F	GAGCACTGGAGAGAAAGGATTTG	124–146	AF228021	71	60
CYCLO-R	GGCACATAAATCCCGGAATTATT	194–172			
GAPDH-F	TGACCCCTTCATTGACCTTCA	66–86	BTU85042	66	60
GAPDH-R	AACCTGCCGTGGGTGGAAT	131–113			
GNMT-F	GTGACAAGATGCTGAAGTATGCTCTC	263–288	XM_618409	71	59
GNMT-R	CCACTTGCTCAAAGGCAGGGT	333–314			
GUSB-F	ATCGGCAGCGCCCACTACT	414–432	NM_001083436	65	60
GUSB-R	CCCTCGTGCTCTAACACATGGAC	478–456			
MUT-F	CGAATTGCCAGGAACACACA	1392–1411	NM_173939	70	59
MUT-R	CCCAAGGATCAGCCACTTTG	1461–1442			
MS-F	CAAATCCATAAGGAGTACCTCCTAGC	259–284	DQ084519	76	59
MS-R	GGGCAATAGAAGTGCTGCTGA	334–313			
MTHFR-F	CATCATCACCAGCTATTCTTTGA	860–883	AY854631	76	59
MTHFR-R	GGTATGCCATCTCTCGGAGCAA	935–914			
MTP-F	GGTCCGTCAAGTTCTGAAGGAA	1757–1778	BRTMTP1	69	59
MTP-R	GAGGAGGACCCACTCTTGAG	1825–1805			
MTRR-F	TCTTCTGATAGAGTTGGACATCTCGA	884–909	DQ084520	80	59
MTRR-R	TGTTGGGACAGATCACGTTGA	963–943			
PEMT-F	GCTGTCCTTGCCATTGTCTTC	513–533	NM_182989	69	58
PEMT-R	TCGGGTCTTGTTGCCATC	581–562			

¹ACTB = β -actin; BHMT = betaine homocysteine methyltransferase; CYCLO = cyclophilin; GNMT = glycine N-methyltransferase; GUSB = β -glucuronidase; MUT = methylmalonyl-CoA mutase; MS = methionine synthase; MTHFR = 5,10-methylene-tetrahydrofolate reductase; MTP = microsomal transfer protein; MTRR = methionine synthase reductase; and PEMT = phosphatidylethanolamine methyltransferase. F = forward primer; R = reverse primer.

²Bold font indicates degenerate primers where M = A or C; R = A or G; Y = C or T; K = T or G; S = C or G.

³RACE = random amplification of cDNA ends.

drofolate reductase (**MTHFR**), betaine homocysteine methyltransferase (**BHMT**), and methionine synthase reductase (**MTRR**), degenerate primers (Table 1) were designed based on homology between human (GenBank accession nos. NM_005957, BC012616, and AF025794, respectively) and mouse (GenBank accession nos. NM_010840, AF033381, and BC025942, respectively) sequences.

The PCR amplifications were performed under the following conditions: the 50- μ L PCR reaction contained 200 μ M dNTP, 300 nM of each primer, 1.5 mM MgCl₂, 1 unit of *Taq* polymerase (Clontech, a Takara Company, Mountain View, CA) in 1 $\times$ *Taq* polymerase buffer. The

PCR profile consisted of an initial denaturation step at 94°C for 1 min followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, 68°C for 1 min, and a final extension at 68°C for 5 min. This amplification generated one fragment of 612, 489, and 516 bp for MTHFR, BHMT, and MTRR, respectively (Table 1). The nucleotide sequence of the amplified fragment was determined by cycle sequencing in both directions for a total of 3 independent amplifications. Sequence determination was performed using the Big Dye Terminator Cycle Sequencing Ready Reactions (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and run on an ABI 377 DNA sequencer (PE Applied Biosystems).

5'- and 3'-Rapid Amplification of the cDNA Ends of MTHFR, BHMT, MTRR, and Methionine Synthase Bovine Genes. To obtain the complete coding sequence of bovine MTHFR, BHMT, MTRR, and methionine synthase (MS), 5' and 3' rapid amplification of the cDNA ends (**RACE**) was performed using the Marathon cDNA amplification kit (Clontech). Poly-A⁺ RNA was isolated using the Nucleotrap mRNA purification kit (Clontech). One microgram of poly-A⁺ RNA from bovine liver tissue was reverse transcribed using the Marathon cDNA synthesis primer. Second strand of cDNA was synthesized and a Marathon cDNA adaptor (Clontech) was then ligated to it. The 3' and 5' end amplification of selected bovine genes was performed using gene-specific primers, defined in Table 1. Both 5' and 3' end fragments were sequenced as described above and were assembled using the AutoAssembler 2.0 software (PE Applied Biosystems) to determine the complete bovine-specific MTHFR, BHMT, MTRR, and MS coding sequences.

mRNA Levels of Selected Bovine Genes. The cDNA of cells obtained by liver biopsy was analyzed for β -actin (**ACTB**), BHMT, cyclophilin (**CYCLO**), GAPDH, glucuronidase- β (**GUSB**), glycine N-methyltransferase (**GNMT**), MS, MTHFR, microsomal transfer protein (**MTP**), MTRR, MUT, and phosphatidylethanolamine methyltransferase (**PEMT**) mRNA levels using real-time PCR amplification. Gene-specific primers (Table 1) were designed and selected using the Primer Express Software (PE Applied Biosystem). Real-time PCR amplifications were performed in 25- μ L reaction volumes consisting of 300 nM forward and reverse primers, 1 μ L of cDNA, 0.25 AmpErase (PE Applied Biosystem), and 1 $\times$ SYBRGreen Master Mix (PE Applied Biosystem). Cycling conditions were 2 min at 50°C, followed by 10 min at 95°C, then 40 cycles of 15 s at 95°C, and 1 min at annealing temperature (Table 1). Amplification, detection, and analysis were performed with an ABI Prism 7700 Sequence Detector (PE Applied Biosystem). A correction for each plate was achieved by dividing the threshold cycle value of samples by the smallest threshold cycle value of the plate. Then, samples were normalized using gene expression of GAPDH as the housekeeping gene because it was not significantly ($P \geq 0.08$) affected by the treatments. Rhoads et al. (2003) and Janovick-Guretzky et al. (2007) observed that, during the peripartum period in dairy cows, expression of many genes frequently used as housekeeping genes varied. Therefore, in the present experiment, gene expression of ACTB, CYCLO, and GUSB was also measured to normalize the data using the "bestkeeper" gene according to Vandesompele et al. (2002). However, gene expression of these 3 genes and consequently of the "bestkeeper" gene was significantly

affected by the studied treatments (Met $\times$ vitamins interaction, $P < 0.0001$).

All real-time PCR reactions were performed in triplicate and standard curves were established in duplicate for each gene. A pool of cDNA obtained from different liver biopsies was used to create a standard curve for quantification of the transcripts using the relative standard curve method as described by Applied Biosystem (1997; User Bulletin #2). Standard curve arbitrary units were made using 2, 1.5, and 1 μ L and then serial dilutions corresponding to 0.75, 0.50, 0.25, 0.10, 0.05, 0.025, and 0.005 μ L of cDNA. For each sample, the amount of each target gene mRNA relative to endogenous GAPDH was determined from their respective standard curves. Relative quantity ratios were obtained by dividing the relative quantity unit of each target gene by that of GAPDH. The specificity of the amplified fragments was verified on a 3.5% agarose gel and with melting curve analysis using the Dissociation Curves v1.0 software (PE Applied Biosystem).

Statistical Analysis

Two levels of Met supplementation (0 or 18 g of RPM) and 3 supplements of B-vitamins (none, folic acid alone or in combination with vitamin B₁₂) were used in a 2 $\times$ 3 factorial arrangement in a 10-block randomized complete block design. All variables were analyzed using the MIXED procedure of SAS (SAS Institute, 2004) according to a complete block design with treatments as fixed effects, repeated measures in time, and block as random effect. Because the time intervals were unequally spaced, the following covariance structures were compared: SP(POW), SP(GAU), SP(EXP), SP(LIN), SP(LINL), and SP(SPH). For each variable, the statistical analysis retained was the one with the smallest fit statistic values. Results are reported as least squares means and standard errors of the means. Means were assumed to be different at $P \leq 0.05$ and tended to differ at $0.05 < P \leq 0.10$. When the vitamin effect had $P \leq 0.10$, differences between means were compared using an adjusted Tukey test. When the Met $\times$ vitamin interaction or a treatment $\times$ time interaction reached a level of significance of 90%, the SLICE option in the LSMEANS statement was used to help interpretation (SAS Institute, 2004).

RESULTS

B-Vitamins

Hepatic concentrations of folates were increased (vitamin effect, $P = 0.001$) with intramuscular injections of folic acid alone or combined with vitamin B₁₂; 6.7,

Table 2. Effects of dietary supplements of rumen-protected methionine (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows from 3 wk before calving to 16 wk of lactation on liver concentrations of B vitamins, lipid fractions, and relative mRNA abundance of selected genes

Item	Treatment ¹						SEM	P-value		
	M+			M–				Met	Vit	Met × Vit
	B ₉ –B ₁₂ –	B ₉ +B ₁₂ –	B ₉ +B ₁₂ +	B ₉ –B ₁₂ –	B ₉ +B ₁₂ –	B ₉ +B ₁₂ +				
Cows (n)	10	10	10	10	10	10				
Vitamins (μg/g of fresh tissue)										
Folates ²	6.4	10.2	10.0	7.0	10.5	10.8	0.4	0.03	0.001	0.63
Vitamin B ₁₂	0.91	0.91	1.05	0.98	0.89	1.15	0.03	0.004	0.001	0.03
Lipids (g/100 g of fresh tissue)										
Cholesterol ester ³	0.112	0.113	0.125	0.111	0.096	0.112	0.012	0.21	0.27	0.61
Triglycerides ³	3.08	1.96	2.86	1.53	1.88	2.02	0.49	0.02	0.46	0.24
Diglycerides, fraction 1 ³	0.080	0.077	0.090	0.080	0.073	0.090	0.010	0.86	0.18	0.95
Diglycerides, fraction 2 ³	0.481	0.434	0.480	0.470	0.467	0.477	0.028	0.77	0.51	0.68
Cholesterol ³	0.179	0.190	0.194	0.192	0.173	0.181	0.009	0.42	0.79	0.11
Phosphatidylethanolamine (PE) ³	0.768	0.742	0.739	0.751	0.728	0.761	0.023	0.87	0.54	0.64
Phosphatidylcholine (PC) ³	1.68	1.71	1.66	1.67	1.68	1.70	0.06	0.92	0.94	0.81
Total lipids ³	5.03	4.39	4.84	4.47	4.31	4.46	0.24	0.07	0.18	0.55
PC/PE ratio ³	2.20	2.33	2.26	2.22	2.32	2.25	0.05	0.98	0.08	0.91
Relative mRNA abundance ⁴										
BHMT ⁵	0.98	0.97	1.07	0.86	0.97	0.94	0.09	0.17	0.52	0.61
GNMT ⁶	0.48	0.75	0.73	0.55	0.70	0.70	0.15	0.99	0.19	0.87
MS ³	0.84	0.89	0.96	0.81	0.92	0.78	0.07	0.26	0.45	0.23
MTHFR ⁵	0.95	0.96	1.08	0.73	0.88	0.98	0.08	0.02	0.02	0.47
MTP ⁵	1.2	1.16	1.24	1.03	1.16	1.12	0.11	0.05	0.60	0.35
MTRR	0.96	1.02	1.03	0.9	0.94	0.98	0.10	0.19	0.44	0.98
MUT	1.1	1.12	1.16	0.93	1.12	1.18	0.09	0.20	0.01	0.16
PEMT ³	1.15	1.19	1.17	1.05	1.14	1.12	0.07	0.10	0.48	0.83

¹M–: basal diet; M+: basal diet with rumen-protected Met; B₉–B₁₂–: no vitamin supplement; B₉+B₁₂–: 160 mg folic acid/wk; B₉+B₁₂+: 160 mg folic acid/wk + 10 mg of cyanocobalamin/wk.

²Methionine × vitamin × time, $P = 0.002$.

³Time effect (2, 4, 8, and 16 wk of lactation), $P \leq 0.04$.

⁴Relative mRNA abundance of selected genes normalized with GAPDH as housekeeping gene. BHMT = betaine homocysteine methyltransferase; GNMT = glycine N-methyltransferase; MUT = methylmalonyl-CoA mutase; MS = methionine synthase; MTHFR = 5,10-methylene-tetrahydrofolate reductase; MTP = microsomal transfer protein; MTRR = methionine synthase reductase; and PEMT = phosphatidylethanolamine methyltransferase.

⁵Methionine × time, $P \leq 0.10$.

⁶Vitamin × time, $P \leq 0.06$.

10.3, and 10.4 ± 0.28 μg/g of fresh tissue for B₉–B₁₂–, B₉+B₁₂–, and B₉+B₁₂+ cows, respectively (Table 2). Rumen-protected Met decreased ($P = 0.03$) folate concentrations from 9.4 to 8.9 ± 0.25 μg/g of fresh tissue. The effect of B-vitamins and Met supplementation on liver folates also changed during lactation (Met × vitamin × time interaction, $P = 0.002$; Figure 2). Hepatic concentrations of folates generally increased from 2 wk after calving to reach a plateau at 8 wk of lactation, except for M–B₉+B₁₂+ cows, for which a plateau was reached earlier, at 4 wk of lactation.

Hepatic concentrations of vitamin B₁₂ were increased by vitamin B₁₂ supplements; 0.94 , 0.90 , and 1.10 ± 0.02 μg/g of fresh tissue for B₉–B₁₂–, B₉+B₁₂–, and B₉+B₁₂+ cows, respectively (vitamin effect, $P = 0.001$). Nevertheless, Met supply modified the response to vitamin supplementation (Met × vitamin interaction, $P = 0.03$; Table 2). For control cows ($P = 0.02$) and cows

injected with folic acid and vitamin B₁₂ together ($P = 0.002$), liver concentrations of vitamin B₁₂ were higher in M– than M+ cows, whereas there was no such effect in cows injected with folic acid alone ($P = 0.63$).

Lipids

Hepatic concentrations of total lipids decreased as lactation progressed (time effect, $P = 0.0001$), from 6.29, 4.93, 3.72, to 3.39 ± 0.18 g/100 g of fresh tissue at 2, 4, 8, and 16 wk of lactation, respectively. Hepatic concentrations of CE, TG, DG, Ch, and PE followed a similar pattern (time effect, $P \leq 0.04$); only PC concentrations in liver increased during the experimental period (time effect, $P = 0.0001$, data not shown).

Dietary supplements of RPM increased ($P = 0.02$) liver concentrations of TG from 1.81 to 2.63 ± 0.32 g/100 g of fresh tissue and tended ($P = 0.07$) to in-

Figure 2. Effects of dietary supplements of rumen-protected methionine (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows from 3 wk before calving to 16 wk of lactation on hepatic concentrations of folates. The shaded symbols represent cows fed 18 g of rumen-protected methionine (M+) and the open symbols represent cows without Met supplements (M-). B₉-B₁₂- (Δ, ▲) = no vitamin supplement; B₉+B₁₂- (○, ●) = 160 mg of folic acid/wk; B₉+B₁₂+ (□, ■) = 160 mg of folic acid/wk + 10 mg of vitamin B₁₂/wk (SE = 0.55; Met × vitamin × time, $P = 0.002$).

crease total lipid concentrations from 4.41 to 4.75 ± 0.16 g/100 g of fresh tissue (Table 2). Liver concentrations of CE, DG (fractions 1 and 2), Ch, PE, and PC were not affected by treatments ($P \geq 0.2$; Table 2).

The PC/PE ratio tended ($P = 0.08$) to vary according to the vitamin supplementations, 2.20 , 2.32 , and 2.25 ± 0.04 for B₉-B₁₂-, B₉+B₁₂-, and B₉+B₁₂+ cows, respectively; the ratio being higher ($P \leq 0.07$) for B₉+B₁₂- than for B₉-B₁₂- cows but not different ($P = 0.37$) from B₉+B₁₂+ cows.

Gene Expression of BHMT, GNMT, MS, MTHFR, MTP, MTRR, MUT, and PEMT

Vitamin supplements affected gene expressions of MTHFR (0.84 , 0.92 , and 1.03 ± 0.07 , $P = 0.02$) and MUT (1.02 , 1.12 , and 1.17 ± 0.08 , $P = 0.01$; Table 2) for B₉-B₁₂-, B₉+B₁₂-, and B₉+B₁₂+ cows, respectively. The relative mRNA abundance of MTHFR gene in liver of B₉+B₁₂+ cows was higher ($P = 0.006$) than for B₉-B₁₂- and tended to be higher ($P = 0.1$) than for B₉+B₁₂- cows but the last 2 treatments were not different ($P = 0.3$). Compared with the control treatment, supplementary folic acid combined with vitamin B₁₂ increased ($P = 0.004$) MUT relative mRNA abundance, whereas supplementary folic acid given alone only tended to increase it ($P = 0.06$).

In M- cows, expressions of MTHFR, MTP, and PEMT transcripts were lower by 13 ($P = 0.02$), 8 ($P = 0.05$), and 6% ($P = 0.10$) compared with cows fed the basal diet supplemented with RPM (Table 2). The relative mRNA abundance of MS and MTRR genes was not affected by treatments ($P > 0.2$; Table 2).

Ingestion of RPM modified the relative mRNA abundance of MTP, MTHFR, and BHMT genes according to the stage of lactation (Met × time interaction, $P = 0.10$ for MTP and MTHFR and $P = 0.03$ for BHMT). Overall, the transcript abundance of these genes decreased throughout the experimental period but was lower at 2 and 16 wk of lactation for M- cows. The mRNA levels of GNMT tended to change with time according to vitamin supply (vitamin × time interaction, $P = 0.06$; Figure 3). There was no effect ($P \geq 0.5$) of vitamin supplementation on GNMT relative mRNA abundance in liver at 2, 4, and 8 wk of lactation but it increased ($P = 0.002$) at 16 wk of lactation in cows injected with vitamins, especially in B₉+B₁₂+ cows.

The relative mRNA abundance of PEMT gene decreased throughout the experimental period (time effect, $P < 0.0001$), whereas MS gene transcripts increased from 2 to 8 wk of lactation but decreased thereafter (time effect, $P = 0.0003$). There was no variation of MTRR and MUT transcript abundance throughout the experimental period ($P > 0.11$).

Figure 3. Effects of dietary supplements of rumen-protected methionine (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows from 3 wk before calving to 16 wk of lactation on glycine N-methyltransferase (GNMT) relative mRNA abundance in liver. Gene expression ratio was normalized with GAPDH. B₉-B₁₂- (▲) = no vitamin supplement; B₉+B₁₂- (●) = 160 mg of folic acid/wk; B₉+B₁₂+ (□) = 160 mg of folic acid/wk + 10 mg of vitamin B₁₂/wk (SEM = 0.14; vitamin × time, *P* = 0.06).

DISCUSSION

B-Vitamin Status

In the present experiment, as previously observed by Graulet et al. (2007), folic acid and vitamin B₁₂ supplements increased concentrations of these vitamins in liver of dairy cows. Nevertheless, independent of vitamin supply and as previously observed in steers (Lambert et al., 2002), hepatic concentrations of folates decreased with Met supplementation. Similarly, while studying the effect of severe vitamin B₁₂ deficiency in sheep, Smith et al. (1974) observed that in pair-fed sheep not deficient in vitamin B₁₂, supplementary Met decreased hepatic concentrations of folates, especially 5-methyl-tetrahydrofolate.

In the present experiment, as expected, injections of vitamin B₁₂ increased hepatic concentrations of the vitamin. Nevertheless, supplementary Met decreased concentrations of vitamin B₁₂ in liver of B₉-B₁₂- and B₉+B₁₂+ cows but not in B₉+B₁₂- cows. In cells, vitamin B₁₂ is bound to proteins, mostly the 2 vitamin B₁₂-dependent enzymes MS and MUT (Combs, 1998). Because MS is regulated by vitamin B₁₂ (Oltean and Banerjee, 2003, 2005), it is possible that increasing the supply in preformed labile methyl groups, reducing the demand for remethylation as demonstrated in sheep (Lobley et al., 1996), also decreased cellular retention of vitamin B₁₂. However, there is no clear explanation for the absence of effect of supplementary Met when folic acid supplements were given alone, although Graulet et al. (2007) also observed that supplementary folic acid reduced the increase in concentrations of vitamin B₁₂

in liver of dairy cows fed a diet similar to the M- diet used in the present experiment during the first 8 wk of lactation.

The Methylation Cycle

The role of the methylation cycle is to ensure a constant supply of SAM, which is a methyl donor in numerous metabolic reactions, such as formation of PC and creatine and DNA methylation (Scott, 1999). In this cycle, the role of folate coenzymes is to provide 1-carbon units for de novo formation of methyl groups. Tetrahydrofolate (THF) accepts 1-carbon units and forms 5,10-methylene-THF which, under the action of MTHFR, is irreversibly converted into 5-methyl-THF (Bailey and Gregory, 1999; Figure 1). The enzyme activity is allosterically inhibited by the product of the methylation cycle, SAM (Jencks and Matthews, 1987; Yamada et al., 2005). Consequently, based on the relationship between SAM and MTHFR activity described above, conditions increasing methyl group supply likely inhibit enzyme activity. In the present experiment, however, increasing the supply of labile methyl groups, preformed (RPM) or by promoting methylneogenesis (folic acid and vitamin B₁₂ supplements), increased the mRNA abundance of MTHFR, but MTHFR activity and protein level were not measured.

The second reaction related to the methylation cycle involves the enzyme MS. In this reaction, the 5-methyl-THF resulting from the MTHFR reaction transfers its methyl group to the vitamin B₁₂ coenzyme, which acts as an intermediary transporter to transfer this group

to Hcy, thus forming Met (Figure 1). In the present experiment, neither vitamin supplements nor RPM affected gene expression of MS. In dairy cows, Graulet et al. (2007) also did not observe changes in mRNA abundance and enzymatic activity of MS in response to dietary supplements of folic and vitamin B₁₂, given alone or in combination. This is in agreement with observations from Gulati et al. (1999) who demonstrated that regulation of MS activity is not a function of changes in mRNA abundance or conversion of the apoenzyme into the holoenzyme. Rather, the regulation of MS occurs through improved translational efficiency modulated by vitamin B₁₂ as also recently reported by Olteanu and Banerjee (2003; 2005).

A functional deficiency in MS can also occur. Indeed, as described previously, MS catalyzes the transfer of the methyl group from 5-methyl-THF to the vitamin B₁₂ cofactor and then to Hcy for regeneration of Met and THF. The vitamin B₁₂ cofactor is under the form of cob(I)alamin but, after successive cyclings, it may be oxidized in cob(II)alamin, causing the inactivation of MS. Regeneration of the enzyme activity requires a reductive methylation, which is catalyzed by MTRR and uses SAM as a methyl group donor (Olteanu and Banerjee, 2001). In the present study, neither treatment nor time after calving affected gene expression of MTRR.

Remethylation of Hcy can also occur through BHMT, a methyltransferase that catalyzes the transfer of a methyl group from betaine, a product of choline oxidation, to Hcy for Met regeneration (Figure 1). An *in vitro* study suggests that, in rat liver, MS and BHMT contributed equally to the remethylation of Hcy (Finkelstein and Martin, 1984). In rat liver, the response of BHMT activity to Met restriction and dietary supply of other methyl donors is caused by changes in gene expression of the enzyme (Park and Garrow, 1999). Nevertheless, in ruminants, regulation of BHMT activity differs markedly from that in nonruminants. In dairy ruminants, availability of choline from dietary supply is low because of its extensive degradation in rumen (Sharma et al., 1989), whereas the output of methylated compounds to milk is particularly high. Xue and Snoswell (1986) demonstrated that the activity of BHMT is more than 5 times lower in liver of sheep than rats. Moreover, the activity of this enzyme is not increased during lactation in ewes in spite of the increased demand for methyl groups (Xue and Snoswell, 1985). Similarly, there was no recovery of labeled choline in the Met pool of lactating goats, indicating that in ruminants, the role of BHMT would be negligible for Hcy remethylation (Emmanuel and Kennelly, 1984). In growing steers, BHMT activity changed quadratically with increased Met supply, the higher activity being

observed with the lowest and the highest Met supply (Lambert et al., 2002). In the present experiment, gene expression of BHMT was not influenced markedly by treatments although it was higher in cows fed RPM at 2 and 16 wk after calving. Even assuming that changes in gene expression of the enzyme are directly related to its activity in cows, given that plasma concentrations of Hcy were not affected by RPM (Preynat et al., 2009a) and the limited role of BHMT in the transmethylation pathway in ruminants, the physiological significance of these changes is difficult to assess.

In nonruminants, the major reactions of methylation are synthesis of PC through PEMT, creatine through guanidinoacetate N-methyltransferase, and sarcosine through GNMT (Mudd et al., 2007). Utilization of methyl groups by the former 2 methyltransferases is limited by the physiological need for their product but that is not the case for GNMT, which acts as a safety valve to avoid methyl group excess (Luka et al., 2007). In the present experiment, to study the effect of increasing provision of preformed labile methyl groups or promoting methylneogenesis by folic acid and vitamin B₁₂ supply, gene expression of PEMT and GNMT was studied.

Through 3 successive methylations, PEMT allows PE to form PC (Vance et al., 2007). Phosphatidylcholine is essential for the structural integrity of cell membranes, is secreted into bile, and is a constituent of plasma lipoproteins (Jacobs et al., 2005), this last function being important in lactating ruminants because of the increased demand for milk fat synthesis (Emmanuel and Kennelly, 1984). In the present study, the level of PEMT mRNA tended to increase with Met supplements. Jacobs et al. (2005) reported that Met supply affects PEMT gene expression and activity. In addition, it appears that increased gene expression of PEMT is associated with an increase in protein abundance and enzyme activity (Resseguie et al., 2007). However, in the current study, even if the trends of RPM to increase gene expression of PEMT could have been associated with changes in the enzyme activity, hepatic concentrations of PE and PC as well as the PC:PE ratio were not affected by supplementary Met.

The role of GNMT is to maintain a constant ratio of SAM to S-adenosylhomocysteine. When methyl group supply is low, GNMT is inhibited to conserve methyl groups for metabolically important methylation reactions (Finkelstein, 1990). Sarcosine, the final product of GNMT reaction, has no known metabolic function but it can be rapidly metabolized to provide an 1-carbon unit to form 5,10-methylene-THF (Brosnan and Brosnan, 2006). Indeed, in liver, Met availability from the diet and from the folate-dependent 1-carbon pool modulates, by regulation at the protein level, GNMT

activity directly via SAM (Rowling et al., 2002) and indirectly through 5-methyl-THF (Balaghi et al., 1993; Luka et al., 2007). Glycine N-methyltransferase is also a major folate-binding protein in liver (Luka, 2008). In ewes, lactation increases PEMT activity while decreasing GNMT activity (Xue and Snoswell, 1985), although the latter probably has less physiological importance in ruminants because of the low dietary supply in methyl groups. Indeed, GNMT activity in sheep liver is less than 0.5% of its activity in rat liver (Xue and Snoswell, 1986). Nieman et al. (2006) showed that mRNA and protein abundance of GNMT were related to its enzymatic activity. In the present experiment, Met supply, even in M+ cows, was not sufficient to increase GNMT gene expression. Therefore, the increase in GNMT mRNA abundance observed in cows receiving folic acid with or without vitamin B₁₂ at the end of the studied period was more likely related to the role of this enzyme as a folate-binding protein in liver.

Nevertheless, in the present experiment, in spite of some effects of RPM supplements on gene expression of enzymes involved in the methylation cycle such as MTHFR, BHMT, and PEMT, increasing Met supply had no effect on plasma concentrations of Hcy and Cys reported by Preynat et al. (2009a). However, the decrease in plasma concentrations of Hcy without effect on plasma concentrations of Cys, induced by injections of folic acid alone or combined with vitamin B₁₂ and irrespective of the level of Met added to the diet (Preynat et al., 2009a), as well as the increase of the PC:PE ratio in liver, indicated that folic acid supply altered the efficiency of the methylation cycle. Because supplementary vitamin B₁₂ had no additional effect on plasma Hcy or Cys, supply of this vitamin in the current study was likely sufficient to not negatively interfere with this metabolic pathway. Nevertheless, in the cows used in the present experiment, RPM and folic acid supplements alone had no effect on milk and milk component yields, whereas a combined supplement of the 2 vitamins increased milk component yields (Preynat et al., 2009a). Consequently, it seems that the effects of Met supply and supplementary folic acid on the methylation cycle did not affect lactational performance of dairy cows.

Energy and Protein Metabolism

In early lactation, the increased energy requirements for maintenance and lactation during a period when intake is not optimal contribute to an intensive body fat mobilization, which increases hepatic concentrations of total lipids and TG (Bobe et al., 2004). Another contributing factor to the accumulation of TG in dairy cow liver is the slow rate of TG export as very low density

lipoprotein (**VLDL**). The limited VLDL secretion by bovine liver could be caused by a limiting step in VLDL assembly (Graulet et al., 1998) or, as hypothesized by Bauchart (1993), to a lack of availability of VLDL constituents. Methionine could be a limiting nutrient for hepatic metabolism of fatty acids during the periparturient period as a limiting AA for apolipoprotein synthesis or as a methyl donor for PC synthesis, an essential component of VLDL particles (Grummer, 1993; Noga et al., 2002; Shibano and Kawamura, 2005). As stated by Bernabucci et al. (2004), synthesis of VLDL requires TG, PL, Ch, CE, apolipoprotein (B₁₀₀, C, and E) and the enzyme MTP, which plays a fundamental role in coordinating the assembly of VLDL. Although, in the present experiment, supplementary Met increased gene expression of MTP, this change should be interpreted with caution because in periparturient cows developing fatty liver, the increase of MTP mRNA was not correlated with liver TG, plasma NEFA, or VLDL particles and MTP protein abundance and activity (Bremner et al., 2000; Bernabucci et al., 2004).

In the present study, supplementary Met also increased hepatic concentrations of TG. However, the extent of this increase was somewhat limited as hepatic concentrations of 6.2 and 3.8 g of TG per 100 g of fresh tissue were measured in M+ cows, at 2 and 4 wk of lactation, respectively, which corresponds to a mild fatty liver (Bobe et al., 2004). Moreover, from 8 wk after calving, liver TG concentrations decreased below 1 g of TG per 100 g of fresh tissue (0.6 and 0.3 g of TG per 100 g of fresh tissue at 8 and 16 wk of lactation, respectively, in cows fed RPM), considered the limit for normal liver (Bobe et al., 2004). The slightly higher accumulation of fatty acids in liver of M+ cows is in accordance with a small loss of BW in early lactation and the decrease in plasma glucose (Preynat et al., 2009a).

A previous study showed that in early lactation, supplementary folic acid given alone or in combination with vitamin B₁₂ increased milk and milk protein yields without any effect on DMI (Graulet et al., 2007). However, cows fed supplementary folic acid alone had higher hepatic concentrations of lipids than those fed folic acid and vitamin B₁₂ together. The authors hypothesized that this effect could be related to the vitamin B₁₂-dependent enzyme MUT. This enzyme isomerizes methylmalonyl-CoA (**MMCoA**) in succinyl-CoA and is essential for the entry of propionate in the Krebs cycle and its utilization for gluconeogenesis (Le Grusse and Watier, 1993; Figure 1). Moreover, MMCoA acts as an interface in the regulation of gluconeogenesis and β -oxidation (Zammit, 1990). When vitamin B₁₂ supply is not sufficient, isomerization of MMCoA is decreased, leading to its accumulation, which, even if gluconeogenesis is reduced, inhibits β -oxidation of fatty acids, and

thus promoting lipid accumulation in liver. However, in the present experiment, in contrast to Graulet et al. (2007), a combined supplement of folic acid and vitamin B₁₂ had no effect on hepatic concentrations of TG or total lipids compared with cows fed supplementary folic acid alone.

Vitamin B₁₂ deficiency decreased the MUT mRNA level in liver of rats fed a diet that provided an adequate folic acid supply (Nakao et al., 2009). In the present experiment, gene expression of MUT was increased by B-vitamin supplements compared with unsupplemented cows, and this increase was greater in cows injected with the 2 vitamins together. At 12 wk of lactation, whole-body flux of glucose tended to be higher in cows injected with folic acid and vitamin B₁₂ together compared with control cows, this increase being of a similar size as the observed increment in milk lactose yield (Preynat et al., 2009b). Although folic acid is not known to participate in the MUT pathway, Graulet et al. (2007) observed no effect of supplementary folic acid or vitamin B₁₂ on gene expression of MUT or its activity in cow liver. However, these authors reported that the requirement of the enzyme for its cofactor, vitamin B₁₂, decreased when the 2 vitamins were given together compared with folic acid or vitamin B₁₂ given separately, improving the efficiency of this metabolic reaction. Selhub et al. (2007) observed in humans that when the supply in vitamin B₁₂ is considered adequate, plasma concentrations of methylmalonic acid are inversely proportional to folate supply. In cell culture, inactivation of MS reduces MUT holoactivity by decreasing cellular concentrations of adenosylcobalamin, the vitamin B₁₂ cofactor for MUT (Riedel et al., 1999). These observations indicate that the effect of folic acid supply on MUT was possibly linked to a coordination of the distribution of cobalamin cofactor between cytosol (MS) and mitochondria (MUT). Although the effects of vitamin B₁₂ given alone were not studied in the present experiment, the observed effects of supplementary folic acid and vitamin B₁₂ given together on mRNA abundance of MUT as well as on whole-body flux of glucose at 12 wk of lactation (Preynat et al., 2009b) indicate that administration of the 2 vitamins together could improve gluconeogenesis efficiency.

CONCLUSIONS

In the current study, gene expressions of enzymes closely involved in the methylation cycle were modified by Met supply. Cellular retention of folates and vitamin B₁₂ in liver was also modified when the supply in labile preformed methyl groups was limited. Supplementary folic acid and vitamin B₁₂ also affect MUT mRNA abundance, an enzyme related to gluconeogenesis. In

the cows studied in the present experiment, Preynat et al. (2009a) observed that supplementary folic acid, but not vitamin B₁₂ or RPM, decreased plasma concentrations of Hcy although there was no effect of folic acid given alone or RPM on lactational performance. These observations combined with those of the present experiment indicate that an increase in methyl group supply, preformed or from methylneogenesis, even if it affected efficiency of the methylation cycle, had by itself a limited effect on milk component yields. Therefore, the increases in milk production and milk component yields reported in cows supplemented with folic acid and vitamin B₁₂ together (Preynat et al., 2009a) seem to be related to the effect of the vitamins on the increased gene expression of MUT. This observation is further supported by the increased in whole-body flux of glucose observed in cows supplemented with folic acid and vitamin B₁₂ together compared with unsupplemented cows (Preynat et al., 2009b). Therefore, it seems that the effects of the combined supplement of vitamins on lactational performance were caused by an effect on energy metabolism rather than on methylneogenesis.

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