Effects of supplementation with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester on splanchnic amino acid metabolism and essential amino acid mobilization in postpartum transition Holstein cows

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ABSTRACT

The present study aimed to investigate the effects of 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi) supplementation on splanchnic AA metabolism, essential AA (EAA) mobilization, and plasma AA status in postpartum transition dairy cows. The EAA mobilization was calculated by difference: EAA excretion in milk protein – net portal absorption of EAA or net splanchnic release of EAA. Eight Holstein cows fitted with permanent indwelling catheters in the hepatic portal vein, hepatic vein, mesenteric vein, and an artery in the dry period preceding second parturition were used in the study. Cows were randomly allocated to 1 of 4 treatments in a 2×2 factorial arrangement with factor 1: control (calcium carbonate) versus HMBi [1.5 g of HMBi/kg of dry matter (DM)] and factor 2: high dietary ethanol (19 g/kg of DM) versus high dietary propanol (16 g/kg of DM). Only factor 1 data are presented. Treatments were administered in 4 total mixed rations and initiated on the day of parturition. Cows were sampled 14 d before expected parturition and 4, 15, and 29 d after parturition. Supplementation with HMBi tended to increase milk fat content but not fat yield, tended to impose a slower rate of decrease in milk casein content with increasing days in milk (DIM), prevented the decrease in plasma Met associated with parturition for control, reduced plasma concentration of Ser, tended to reduce plasma concentrations of Gly and His, and tended to increase hepatic uptake of Met postpartum. Cows excreted 248 ± 18 g more EAA in the milk at 4 DIM than was released from splanchnic tissues. The EAA deficiency decreased as lactation progressed and was not affected by HMBi supplementation. It was estimated that $4,700 \pm 600$ g of EAA from extra-splanchnic tissues were secreted in milk protein during the first 29 DIM. Extra-splanchnic EAA mobilization can be crucial to sustain milk protein

yield in the postpartum transition period and HMBi is a fast-working Met source that can improve Met status of postpartum transition cows.

Key words: transition dairy cow, amino acid, metabolism, methionine hydroxy analog

INTRODUCTION

The immediate metabolic adaptation to milk secretion in postpartum transition dairy cows induces mobilization of nutrients deposited in body tissue (Gibb et al., 1992; Andrew et al., 1994). The increased susceptibility for metabolic disorders also characterizing the transition period (Grummer, 1995) has led to extensive studies of the links between excessive lipid mobilization and metabolic diseases (Drackley, 1999). A connection between negative protein balance and reduced health and longevity of cows has likewise been speculated (Bell et al., 2000), but the protein metabolism of the transition cow is rarely investigated. The homeorhetic adaptations associated with lactogenesis involve repartitioning of AA to sustain increased demands of the mammary gland. It has previously been speculated that the essential AA (EAA) excreted in milk protein in excess of the dietary supply during the postpartum transition period were partly of splanchnic origin (Bell et al., 2000). Contrary to this assumption, Larsen and Kristensen (2009) observed that the output of EAA in milk protein was twice the splanchnic EAA release, indicating that peripheral tissues contributed substantially to EAA secretion in milk postpartum. Data on splanchnic EAA fluxes hereby supported estimates based on other techniques in that mobilization of AA can be of fundamental importance for meeting the mammary demand for EAA in the first week of lactation (Komaragiri and Erdman, 1997; Phillips et al., 2003). For that reason, milk protein production as well as several nonmammary functions of AA (immune system, AA-derived regulatory compounds, methyl donation reactions, and protein turnover) might be negatively affected by the shortage of EAA in very early lactation.

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Increasing the supply of metabolizable Met by feeding rumen-protected Met (Berthiaume et al., 2006) or supplying a Met analog [2-hydroxy-4-(methylthio)butanoic acid isopropyl ester, **HMBi**; St-Pierre and Sylvester, 2005; Ordway et al., 2009] have repeatedly been shown to increase milk protein content and yield. This indicates an effect of Met on the postabsorptive metabolism of other AA and on their efficiency of incorporation into milk protein. Methionine is also a key intermediate for methyl group transfer and is involved in numerous pathways (Lobley et al., 1996), including phospholipid synthesis, which might be part of the explanation for the observed increase in milk fat production following HMBi supplementation in some studies (e.g., St-Pierre and Sylvester, 2005). Our hypothesis was that supplementation of postpartum transition cows with HMBi would increase the supply of Met to peripheral tissues and potentially alleviate negative consequences of low postpartum Met status on nutrient allocation to milk production. Therefore, the objectives of the study were to investigate effects of postpartum HMBi supplementation on splanchnic AA metabolism in the postpartum transition dairy cow. In addition, we aimed to determine the composition of milk protein to assess whether extra mammary synthesized proteins accounted for a large fraction of EAA in milk not accounted for by splanchnic AA output.

MATERIALS AND METHODS

The present experiment complied with Danish Ministry of Justice Law No. 382 (June 10, 1987), Act No. 726 (September 9, 1993) concerning experiments with animals and care of experimental animals.

Animals and Housing

Eight Danish Holstein cows entering their second lactation were used in the study. Six weeks before expected parturition, cows were surgically prepared with ruminal cannulas and permanent indwelling catheters in the mesenteric vein, hepatic portal vein, hepatic vein, and an artery (Kristensen et al., 2007). The cows were housed in tie stalls with rubber mats, and wood shavings were used as bedding. Cows were milked at 0530 and 1530 h.

Experimental Design and Treatments

The experimental design was a complete randomized design with repeated measurements and 4 treatments in a 2 × 2 factorial arrangement. Factor 1 was control (calcium carbonate) versus HMBi (1.5 g/kg of DM as 2.6 g of MetaSmart/kg of DM; Table 1) and factor

2 was high dietary ethanol (19 g/kg of DM) versus high dietary propanol (16 g/kg of DM). Only factor 1 data are presented. Treatments were administered by 4 different TMR. Treatments were initiated on the day of calving, assigned as +1 DIM. Prepartum, all cows were offered the same nonlactation ration (Table 1). The feed allowance during the dry period was restricted to 10 kg of DM/d. Postpartum, diets were offered *ad libitum* (10% orts). The daily ration was divided in 3 equivalent meals, fed at 0800, 1600, and 2400 h, and orts removed at 0730 h. Each treatment TMR was mixed in one single mix, bagged in portions of 15 to 20 kg within 2 to 3 h after mixing, and evacuated using a chamber vacuum packaging machine (Webomatic I22, Webo A/S, Årup, Denmark). The vacuum-packed TMR was initially frozen at −20 to −25°C and kept at −6°C during the study period. Feed was allowed to thaw overnight on the barn floor.

Experimental Samplings

Cows were sampled once prepartum at d −15 ± 2 relative to parturition and sampled postpartum on 4, 15, and 29 DIM. Splanchnic blood plasma flows were measured by downstream dilution of *p*-aminohippuric acid (**pAH**; 175 mmol/L sterile solution at pH 7.4) infused continuously into the mesenteric vein at 27.4 ± 1.6 mmol/h, initiated 1 h before the first sampling.

First blood sampling was 30 min before feeding at 0800 h, and a total of 8 hourly sample sets were collected from the artery, portal vein, and hepatic vein. Thirty milliliters of blood was simultaneously collected from each catheter at each sampling time and blood was immediately transferred to heparin vacuettes (Greiner BioOne GmbH, Frickenhausen, Austria). Blood samples were immediately placed on ice and plasma was harvested after centrifugation at 3,000 × *g* for 20 min at 4°C. Plasma was stored at −20°C until analysis. Milk yield and feed intake were recorded daily, and milk samples for analysis of milk composition were collected on sampling days. Nine randomly selected vacuum bags from each TMR were collected for feed analysis. Cows were weighed before the afternoon milking on each sampling day.

Chemical Analyses

To correct for the acetylation of **pAH** occurring in the liver (Kristensen et al., 2009), all plasma samples were deacetylated before analysis by combining plasma with an equal volume of 20% TCA, and then incubated at 100°C for 1 h. Subsequently, **pAH** concentrations were determined according to Harvey and Brothers (1962) using a continuous flow analyzer (method US-216-72

Table 1. Ingredients and nutrient composition of nonlactation and lactation (control and HMBi) TMR (g/kg of DM unless otherwise noted)

Item	Nonlactation	Lactation	
		Control	HMBi
Ingredient			
Corn silage ¹	450	400	400
Grass clover silage ²	200	250	250
Barley straw, chopped	118	—	—
Wheat grain, rolled	75	—	—
Whole wheat grain, NaOH treated	—	207	207
Soybean meal, 47% CP	100	100	100
Molasses, sugar beet	10	17.2	17.2
Sugar beet pulp	24	—	—
Mineral and vitamin premixes	13 ³	6 ⁴	6 ⁴
Vitamin E premix ⁵	—	1	1
Vegetable fat ⁶	7.5	10	10
Feed-grade urea	—	2.5	2.5
NaCl	2.5	—	—
MetaSmart, dry ⁷	—	—	2.6
CaCO ₃	—	5.2	2.6
Chromium(III)oxide	—	1	1
Nutrient			
DM (g/kg)	478	411	405
Ash	57	62	63
Crude fat	36	36	36
NDF	358	273	270
CP	144	155	155
Starch (calculated)	203	277	277
MP ⁸	72	89	90
Metabolizable Met ⁸ (% of MP)	2.23	2.17	2.99
Metabolizable Lys ⁸ (% of MP)	7.01	6.68	6.67
NE _L ⁸ (MJ/kg of DM)	6.84	6.99	7.01

¹Chemical composition of corn silage: DM, 363 (g/kg); ash, 31; CP, 87; NDF, 375; starch, 320; in vitro OM digestibility, 72.5%.

²Chemical composition of grass clover silage: DM, 407 (g/kg); ash, 86; CP, 146; NDF, 363; in vitro OM digestibility, 78.8%.

³Premix dry (Gold alfa Bio org Selen, Vitfoss, Gråsten, Denmark) containing 2% Ca, 4% P, 19.2% Mg, 3.6% Na, 8.3% S, 18,182 mg/kg of vitamin E, 91 mg/kg of biotin, 3,636 mg/kg of Mn, 909 mg/kg of Cu, 36 mg/kg of Co, 6,364 mg/kg of Zn, 227 mg/kg of I, 24 mg/kg of Se.

⁴Premix lactation (VM2, Vitfoss) containing 16% Ca, 5% P, 6.5% Mg, 9% Na, 0.5% S, 600 IU/g of vitamin A, 190 IU/g of vitamin D₃, 4,000 IU/g of vitamin E, 4,000 mg/kg of Mn, 1,500 mg/kg of Cu, 25 mg/kg of Co, 4,500 mg/kg of Zn, 225 mg/kg of I, 50 mg/kg of Se.

⁵Suplex D-alfa E-50000 (Vitfoss) containing 33.557 mg/kg of α -tocopherol.

⁶Palm fatty acid distillate (Scanfedt A/S, Fredericia, Denmark).

⁷2-Hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi; MetaSmart, Adisseo, Antony, France).

⁸Calculated at DMI levels of 10 kg of DMI/d prepartum and 20 kg of DMI/d postpartum using the nonadditive Norfor feed evaluation system (Volden, 2011).

Rev.1; Autoanalyzer 3, Seal Analytic Ltd., Burgess Hill, UK).

All laboratory analyses other than pAH analysis were conducted on samples pooled within cow and sampling day. Heparinized plasma samples were analyzed for AA by GC-MS according to the isotope dilution method described by Calder et al. (1999). A working AA standard was prepared from a commercial AA mixture (AAS18; Sigma-Aldrich Denmark A/S, Brøndby, Denmark) with added glutamine (L-glutamine 99%, final concentration 400 μ M; Acros, Geel, Belgium). The internal standard was made from a [U-¹³C/U-¹⁵N] cell-free AA mixture (CNLM-6696-1; Cambridge Isotope Laboratories Inc.,

Andover, MA) with added [¹⁵N₂] urea (no. 316830; Campro Scientific GmbH, Berlin, Germany). Essential AA analyzed were His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val; and non-EAA analyzed were Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr. Total protein determination was performed using the biuret reaction endpoint method (ABX Pentra Total Protein CP; Horiba ABX, Montpellier, France) adapted for use on a Cobas Mira autoanalyzer. Milk samples were analyzed for fat, protein, and lactose by infrared spectroscopy with a MilkoScan 4000 (Foss Electric, Hillerød, Denmark) at a commercial laboratory (Eurofins Steins A/S, Holstebro, Denmark).

Milk Fractionation

Milk samples from morning and afternoon milking were pooled according to milk yield, and the pooled samples were skimmed by centrifugation at $3,500 \times g$ for 20 min at 5°C. The skim milk was separated into noncasein nitrogen (NCN) and NPN. The NCN fraction was prepared by adjusting the pH of the skim milk to 4.6 using 10% acetic acid to precipitate the caseins and centrifuged at $1,000 \times g$ for 10 min at 5°C. The casein was washed 2 times with water, followed by centrifugation at $1,000 \times g$ for 5 min at 5°C, and finally dissolved in 0.1 M citrate. The NPN fraction was prepared by adding 15% TCA to skim milk (4 + 1; vol/vol) to precipitate all proteins, followed by centrifugation at $1,000 \times g$ for 10 min at 5°C; the supernatant contained the NPN fraction.

Nitrogen determination was performed on skim milk (determination of total N content) and on the NCN and NPN fractions. The skim milk and NCN were weighed into tinfoil capsules, 12.5 and 50 μ L, respectively. Twenty-five microliters of NPN was weighed into silver cups and the exact weight was recorded. The samples were dried at 100°C for 30 min and the N content determined according to the Dumas principle by using an elemental analyzer (Flash EA, Thermo Scientific, Copenhagen, Denmark).

Calculations and Statistics

One cow (HMBi treatment) had retained placenta and her data from 4 DIM were removed from the data set. None of the other cows were affected by diseases during the study and all catheters remained functional throughout the study.

The net fluxes (mmol/h) were calculated as the products of plasma flow (L/h) and venous-arterial concentration differences (mmol/L) for the portal-drained viscera (PDV), liver, and the splanchnic bed as described previously (Kristensen et al., 2007). Positive fluxes indicate a net release of a metabolite and negative fluxes indicates a net uptake of a metabolite.

The EAA output in milk protein was calculated using the milk protein yield and tabulated values for EAA composition of milk (g/kg of protein): His: 27; Ile: 59; Leu: 98; Lys: 83; Met: 28; Phe: 49; Thr: 43; Trp: 15; Val: 66 (Swaigood, 1995). The EAA balance was calculated as the difference between milk protein EAA yield/d and splanchnic or PDV net fluxes of EAA/d. The total EAA deficiency was calculated as the mean EAA balance weighed by the time period represented by each sampling (i.e., DIM 4 = 0.31; DIM 15 = 0.45; DIM 29 = 0.24).

The true protein content in milk was determined as the difference between total N and NPN. Casein N content was calculated as the difference between total N and NCN. Whey protein content was calculated as the difference between NCN and NPN. The N content was multiplied by 6.38 to obtain the milk protein content (DePeters and Cant, 1992).

Data were analyzed as a complete randomized design, where cow was considered as a random factor and DIM was considered as repeated measures, analyzed using an autoregressive order 1 covariance structure. The MIXED procedure in SAS (version 9.1, SAS Institute Inc., Cary, NC) was used. Metabolite concentrations, net flux data, DMI, BW, and milk variables were analyzed using a model including the fixed effects of factor 1 (2 levels), factor 2 (2 levels), DIM (3 levels), and the possible interactions. Statistics in Tables 2 to 6 for effects of treatment, DIM, and treatment $\times$ DIM were computed using a data set containing only postpartum data. Differences between treatment means within DIM were separated using Fisher's protected least significant difference test. To test whether treatment affected the difference between prepartum and 4 DIM (named $P_{\text{trans} \times \text{trt}}$ in tables), data including the prepartum observations and the model described above were used for computing Fisher's least significant difference test. Fisher's least significant difference test was also used to test for an overall difference between prepartum and 4 DIM values (named P_{trans} in tables). Data are presented as least squares means $\pm$ residual standard error of the means. Significance was declared at $P \leq 0.05$ and tendencies were considered at $0.05 < P \leq 0.10$.

RESULTS

Feed Intake, Milk Yield, and Milk Composition

Dry matter intake increased from d 15 prepartum to 4 DIM ($P < 0.01$; Table 2) and continued to increase ($P < 0.01$) postpartum without being affected ($P = 0.30$) by HMBi supplementation. A tendency was observed toward a treatment $\times$ DIM interaction for milk yield ($P = 0.10$), which reflected a lower rate of increase in HMBi compared with control. A tendency ($P = 0.08$) was found for a treatment $\times$ DIM interaction for milk protein content, reflecting a more rapid decrease from 4 to 15 DIM in control compared with HMBi. Milk fat and lactose yield increased ($P = 0.05$ and $P < 0.01$) as lactation progressed. Milk fat content tended to be greater for HMBi ($P = 0.07$) compared with control and decreased ($P = 0.02$) for both groups as lactation progressed. A tendency ($P = 0.06$) was observed for an interaction between treatment and DIM for casein con-

Table 2. Dry matter intake, BW, milk yield, milk composition, and milk protein fractions in periparturient Holstein cows supplemented with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi; 1.5 g/kg of DM) or not supplemented (CON) from the day of parturition

Item	Prepartum		$P_{\text{trans} \times \text{trt}}^1$	P_{trans}^2	4 DIM		15 DIM		29 DIM			$P\text{-value}^4$		
	CON	HMBi			CON	HMBi	CON	HMBi	CON	HMBi	SEM ³	Trt	DIM	Trt $\times$ DIM
DMI (kg/d)	10	10	0.77	<0.01	14	15	17	18	20	20	0.44	0.30	<0.01	0.59
Weight (kg)	637	639	0.20	<0.01	604	625	581	575	581	579	16.9	0.96	<0.01	0.14
Yield (kg/d)														
Milk					27.6	27.6	35.6	32.7	40.1	35.8	2.47	0.52	<0.01	0.10
ECM ⁵					31.0	33.6	34.5	34.6	37.7	36.6	1.85	0.90	0.01	0.36
Milk fat					1.26	1.51	1.38	1.44	1.51	1.58	0.08	0.26	0.05	0.31
Milk protein					1.19	1.21	1.12	1.12	1.18	1.07	0.05	0.60	0.22	0.38
Milk lactose					1.27	1.23	1.73	1.64	1.98	1.77	0.13	0.56	<0.01	0.19
Milk composition (g/kg)														
Fat					45	51	39	44	38	48	2.0	0.07	0.02	0.87
Protein					43	41	32	34	29	30	0.9	0.60	<0.01	0.08
Lactose ⁶					46	45	49	50	49	49	0.06	0.38	<0.01	0.47
Protein composition (g/kg)														
True protein					46	42	32	34	29	31	1.60	0.99	<0.01	0.10
Casein					37	36	25	28	23	24	1.30	0.69	<0.01	0.06
Whey					7.8	7.0	5.2	5.1	4.8	5.5	0.60	0.82	0.01	0.65
NPN					1.2	1.4	1.1	1.2	1.3	1.1	0.10	0.89	0.65	0.54

¹Fisher's LSD test for treatment effect on difference between prepartum and 4 DIM.²Fisher's LSD test for prepartum to differ from 4 DIM.³Standard error of the mean (n = 4; except for 4 DIM HMBi where n = 3).⁴Fixed effects when prepartum observations are excluded from data set.⁵Calculated according to Sjaunja et al. (1991).⁶Lactose monohydrate.

tent in milk, which reflected a more rapid decline from 4 to 15 DIM for control compared with HMBi. The whey protein content decreased ($P < 0.01$) from 4 to 29 DIM and was not affected by treatment ($P = 0.82$).

Arterial Variables

The arterial concentration of total EAA decreased ($P < 0.01$; Table 3) from prepartum to 4 DIM and this effect was apparent for most individual EAA except for Met, Thr, and Trp. Postpartum, total EAA tended to increase with DIM, which was especially apparent for Thr and Trp ($P = 0.04$ and $P = 0.01$). A transition $\times$ treatment effect ($P = 0.01$) was observed for arterial Met, reflecting an increase in the concentration from prepartum to 4 DIM in HMBi compared with a decrease in control. Arterial Met tended to be higher in HMBi compared with control postpartum ($P = 0.09$) and although no interaction between treatment and DIM was detected, the effect appeared smaller on 15 and 29 DIM compared with 4 DIM. Supplementation with HMBi affected arterial His from prepartum to 4 DIM ($P = 0.04$), resulting in a greater decrease for HMBi compared with control. The reduced arterial concentration of His at 4 DIM with HMBi tended ($P = 0.10$) to persist through 29 DIM.

Contrary to that of EAA, the arterial concentration of non-EAA increased ($P < 0.01$) from prepartum to 4 DIM. This increase was particularly mediated by increased ($P < 0.01$) arterial concentrations of Gly and Ser. Postpartum, higher concentrations of Gln ($P = 0.02$) and Glu ($P = 0.05$) were observed for HMBi compared with control, particularly on 15 and 29 DIM. Conversely, HMBi supplementation decreased ($P < 0.01$) the arterial concentration of Ser compared with control. Glycine concentrations tended to be affected by treatment $\times$ DIM ($P = 0.07$), reflecting a decreased Gly concentration in HMBi compared with control, especially at 15 DIM. The arterial concentration of total plasma protein decreased ($P < 0.01$) from prepartum to 4 DIM. By 29 DIM, the protein concentration of both treatments had reached prepartum concentrations.

Blood Flows

The portal and hepatic blood plasma flows increased ($P < 0.01$ to $P = 0.02$; Table 3) from prepartum to 4 DIM, but were not affected by treatment ($P = 0.24$ to $P = 0.73$). Postpartum, portal and hepatic blood plasma flows increased ($P = 0.01$) from 4 to 29 DIM and were not affected by treatment.

Net Portal Fluxes

The net portal fluxes for all AA increased ($P < 0.01$ to $P = 0.06$) from prepartum to 4 DIM except

for His ($P = 0.17$), Cys ($P = 0.45$), and Gln ($P = 0.89$). Treatment did not affect ($P = 0.24$ to $P = 0.99$) the prepartum to 4 DIM differences for any of the net portal fluxes of AA (Table 4). Postpartum, none of the individual EAA were affected by treatment but increased ($P < 0.01$ to $P = 0.04$) with increasing DIM for both treatments except for His and Trp, where no changes in net portal fluxes were detected ($P = 0.20$ and $P = 0.21$). Postpartum, Ala had the greatest net portal flux followed by Gly, Leu, and Ser. The net portal flux of total non-EAA was less ($P = 0.03$) for HMBi compared with control, but increased ($P = 0.02$) from 4 to 29 DIM for both treatments. The net portal flux of Gln differed from that of other AA by being negative both pre- and postpartum, and postpartum the net flux of Gln was greater ($P = 0.05$) for control; however, Gln fluxes showed large variation. The net portal flux of Gly was greater ($P < 0.01$) for control compared with HMBi, especially on 15 DIM (control: 45 mmol/h; HMBi: 20 mmol/h).

Net Hepatic Fluxes

For both treatments the net hepatic fluxes of individual EAA and total EAA were generally negative (net uptake) prepartum (Table 5). Apart from Met and Leu, the postpartum net hepatic fluxes of individual EAA were unaffected by treatment ($P = 0.25$ to $P = 0.95$). Postpartum, a tendency ($P = 0.08$) was observed toward a greater net hepatic uptake of Met for HMBi compared with control, and was most pronounced at 4 and 29 DIM. An interaction between treatment and DIM was observed for net hepatic flux of Leu ($P = 0.04$), reflecting that the hepatic uptake of Leu at 4 DIM with HMBi changed to a net release on 15 and 29 DIM in contrast to the increased net uptake in control from 4 to 15 DIM.

The net hepatic flux of most non-EAA changed from prepartum to 4 DIM, especially the net hepatic uptake of Ala, Asn, Gly, and Ser increased ($P < 0.01$ to $P = 0.02$). However, the net hepatic release of Asp and Glu increased ($P < 0.01$) from prepartum to 4 DIM. A tendency ($P = 0.08$) was found for a treatment $\times$ DIM interaction for the net hepatic flux of Ala, reflecting a reduction in net uptake from 4 to 15 DIM for HMBi compared with a more constant net uptake for control as lactation progressed. Glycine and Ser were removed in the greatest quantities by the liver along with Ala, but no treatment effects ($P = 0.24$ to $P = 0.74$) were observed for Gly and Ser. The net hepatic removal of Gly decreased ($P = 0.04$) as lactation progressed, but did not reach prepartum values by 29 DIM. Unlike Gly, Ser was taken up in increasing amounts ($P = 0.05$) by the liver from 4 to 29 DIM. Glutamate was released in

Table 3. Blood plasma flows and arterial concentrations of AA in periparturient Holstein cows supplemented with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi; 1.5 g/kg of DM) or not supplemented (CON) from the day of parturition

Item	Prepartum		$P_{\text{trans}} \times \text{trt}^1$	P_{trans}^2	4 DIM		15 DIM		29 DIM		SEM ³	P -value ⁴		
	CON	HMBi			CON	HMBi	CON	HMBi	CON	HMBi		Trt	DIM	Trt $\times$ DIM
Blood plasma flow (L/h)														
Portal vein	692	670	0.72	<0.01	949	959	1,074	1,049	1,073	1,066	57	0.73	0.01	0.87
Hepatic vein	766	763	0.73	<0.01	1,092	1,067	1,196	1,158	1,218	1,222	44	0.60	0.01	0.71
Hepatic artery	73	93	0.24	0.02	145	112	121	109	145	156	37	0.95	0.12	0.69
Blood plasma ($\mu\text{mol/L}$)														
Essential AA	753	753	0.71	<0.01	540	560	626	626	666	629	50	0.90	0.10	0.80
Branched-chain AA	437	437	0.76	<0.01	288	299	332	351	344	329	29	0.94	0.15	0.74
His	46	46	0.04	<0.01	38	17	39	15	37	21	7	0.10	0.78	0.47
Ile	115	122	0.78	<0.01	88	87	102	113	104	98	10	0.96	0.05	0.34
Leu	118	119	0.96	<0.01	70	70	74	78	76	83	6	0.61	0.29	0.82
Lys	81	76	0.61	<0.01	51	52	60	65	60	64	5	0.59	0.08	0.85
Met	21	20	0.01	0.79	17	23*	17	22	18	19	1	0.09	0.41	0.18
Phe	47	49	0.36	<0.01	36	41	39	38	41	41	3	0.75	0.45	0.46
Thr	87	93	0.44	0.87	82	100	100	101	125	115	10	0.82	0.04	0.47
Trp	35	32	0.21	0.25	28	32	39	35	41	40	3	0.89	0.01	0.37
Val	199	196	0.44	<0.01	131	141	156	160	164	149	14	0.92	0.20	0.61
Nonessential AA	1,064	1,123	0.69	<0.01	1,354	1,384	1,467	1,399	1,355	1,388	31	0.85	0.11	0.24
Ala	164	196	0.76	0.04	144	161	194	185	201	209	10	0.74	<0.01	0.11
Asn	29	32	0.94	0.16	32	34	43	43	48	47	3	0.83	<0.01	0.87
Asp	8	7	0.10	<0.01	4	4	7	5	7	6	0.6	0.29	<0.01	0.22
Cys	111	110	0.67	<0.01	73	74	97	100	102	95	3	0.88	<0.01	0.22
Gln	294	321	0.80	0.38	321	335	265	344*	246	358**	24	0.02	0.38	0.20
Glu	75	78	0.94	<0.01	63	65	63	69	62	73**	2	0.05	0.38	0.18
Gly	204	199	0.69	<0.01	491	466	524	429*	416	368	25	0.14	<0.01	0.07
Pro	59	65	0.40	0.21	61	71	82	81	85	78	4	0.10	<0.01	0.09
Ser	73	72	0.40	<0.01	140	126	157	105**	142	105*	7	<0.01	0.84	0.12
Tyr	45	44	0.29	<0.01	26	30	36	38	45	42	3	0.78	<0.01	0.41
Total AA	1,811	1,876	0.97	0.16	1,894	1,936	2,093	2,025	2,024	2,017	57	0.81	0.04	0.50
Total protein (g/L)	83	78	0.60	<0.01	73	71	79	75	81	81	1.95	0.36	0.01	0.50

¹Fisher's LSD test for treatment effect on difference between prepartum and 4 DIM.²Fisher's LSD test for prepartum to differ from 4 DIM.³Standard error of the mean ($n = 4$; except for 4 DIM HMBi where $n = 3$).⁴Fixed effects when prepartum observations are excluded from data set.* $P \leq 0.05$; ** $P \leq 0.01$ (difference between means within DIM).

Table 4. Net portal fluxes¹ of AA in periparturient Holstein cows supplemented with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi; 1.5 g/kg of DM) or not supplemented (CON) from the day of parturition

Plasma flux (mmol/h)	Prepartum		$P_{\text{trans} \times \text{trt}}$ ²	P_{trans} ³	4 DIM		15 DIM		29 DIM		SEM ⁴	P -value ⁵		
	CON	HMBi			CON	HMBi	CON	HMBi	CON	HMBi		Trt	DIM	Trt $\times$ DIM
Essential AA	72	81	0.52	<0.01	128	150	156	145	193	182	9.70	0.82	<0.01	0.29
Branched-chain AA	31	38	0.73	<0.01	64	74	81	72	97	87	4.20	0.42	<0.01	0.10
His	4.3	3.5	0.24	0.17	4.6	6.6	7.8	6.4	8	8	1.23	0.99	0.20	0.49
Ile	9.3	11	0.75	<0.01	19	22	22	21	28	26	1.63	0.85	<0.01	0.44
Leu	11	14	0.80	<0.01	25	29	32	30	38	34	1.49	0.36	<0.01	0.08
Lys	15	16	0.94	0.02	20	22	25	24	32	31	2.39	0.85	<0.01	0.86
Met	3.6	4.3	0.60	<0.01	6.2	7.5	7	6.8	9.0	9.5	0.58	0.50	<0.01	0.48
Phe	8.5	9.2	0.49	<0.01	14	17	16	16	20	20	1.24	0.55	<0.01	0.52
Thr	8.5	8.6	0.35	<0.01	16	19	16	17	22	22	2.18	0.75	0.04	0.80
Trp	1.4	1.7	0.99	0.02	2.8	3.1	3.2	3.6	4.2	3.8	0.53	0.94	0.21	0.73
Val	10	12	0.64	<0.01	20	24	26	22	32	27	2.11	0.34	0.02	0.16
Nonessential AA	51	75	0.59	<0.01	138	135	183	126	223	185	20.6	0.03	0.02	0.57
Ala	24	27	0.97	<0.01	45	48	55	50	62	58	4.30	0.51	0.04	0.64
Asn	8.4	11	0.91	<0.01	16	18	20	17	21	21	2.20	0.76	0.20	0.51
Asp	1.2	1.2	0.26	0.06	2.7	1.7	2.6	2.2	3.5	3.5	0.85	0.70	0.16	0.82
Cys	2.8	1.7	0.62	0.45	3.6	5.0	1.5	3.5	2.7	0.6	1.30	0.72	0.18	0.30
Gln	-16	-4.5	0.69	0.89	-12	-15	-0.3	-22	34	-0.9	14.9	0.05	0.10	0.59
Glu	-1.7	-1.2	0.92	<0.01	2.4	2.7	4.1	3.1	5.0	4.9	0.99	0.73	0.10	0.81
Gly	8.1	11	0.31	0.01	32	24	45	20**	34	28	4.72	<0.01	0.56	0.35
Pro	4.2	5.6	0.64	<0.01	11	11	14	13	17	15	1.37	0.37	0.01	0.88
Ser	14	16	0.98	<0.01	25	27	27	26	28	38	3.04	0.24	0.10	0.29
Tyr	7.0	8.4	0.36	<0.01	13	13	14	14	16	17	0.93	0.79	0.01	0.83
Total AA	123	155	0.84	<0.01	266	286	339	272	417	367	27.6	0.10	<0.01	0.41

¹Net portal flux was calculated as portal blood plasma flow $\times$ (portal concentration – arterial concentration).²Fisher's LSD test for treatment effect on difference between prepartum and 4 DIM.³Fisher's LSD test for prepartum to differ from 4 DIM.⁴Standard error of the mean ($n = 4$; except for 4 DIM HMBi where $n = 3$).⁵Fixed effects when prepartum observations are excluded from data set.* $P \leq 0.05$; ** $P \leq 0.01$ (difference between means within DIM).

Table 5. Net hepatic fluxes¹ of AA in periparturient Holstein cows supplemented with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi; 1.5 g/kg of DM) or not supplemented (CON) from the day of parturition

Plasma flux (mmol/h)	Prepartum				4 DIM		15 DIM		29 DIM		SEM ⁴	<i>P</i> -value ⁵		
	CON	HMBi	$P_{trans \times trt}$ ²	P_{trans} ³	CON	HMBi	CON	HMBi	CON	HMBi		Trt	DIM	Trt × DIM
Essential AA	-18	-28	0.64	0.73	-22	-28	-34	-13	-33	-21	7.5	0.29	0.82	0.12
Branched-chain AA	1.6	-7.2	0.66	0.96	-0.4	-5.7	-11	1.0	-7.7	1.6	4.0	0.24	0.78	0.08
His	-1.9	-2.3	0.87	0.92	-2.0	-1.9	-3.9	-1.9	-1.2	-3.2	1.6	0.88	0.91	0.58
Ile	1.6	-1.5	0.99	0.41	0.2	-2.9	-2.8	1.0	-2.4	0.2	1.6	0.40	0.99	0.16
Leu	-0.5	-1.8	0.48	0.51	-0.6	-0.2	-4.7	0.1**	-4.0	1.6**	1.0	<0.01	0.23	0.04
Lys	-4.1	-3.5	0.48	0.70	-4.9	-1.3	-3.5	-2.0	-4.9	0.7	2.4	0.25	0.90	0.58
Met	-1.7	-2.6	0.80	0.51	-2.2	-2.9	-2.2	-2.2	-1.7	-3.0	0.5	0.08	0.86	0.58
Phe	-6.3	-6.9	0.65	0.85	-6.1	-7.6	-7.6	-5.6	-8.6	-9.5	0.9	0.95	0.03	0.32
Thr	-4.6	-4.7	0.65	0.17	-6.0	-6.9	-4.9	-0.8	-7.8	-6.1	1.9	0.47	0.01	0.18
Trp	-0.8	-1.1	0.94	0.82	-0.8	-1.3	-1.6	-1.2	-1.4	-1.2	0.6	0.94	0.80	0.80
Val	0.6	-3.9	0.80	0.99	-0.04	-3.5	-3.0	-0.1	-1.2	-0.1	2.7	0.95	0.86	0.18
Nonessential AA	-50	-53	0.77	0.05	-102	-93	-111	-71	-116	-114	18.2	0.29	0.47	0.60
Ala	-22	-23	0.21	<0.01	-48	-57	-48	-42	-45	-49	3.9	0.74	0.14	0.08
Asn	-2.9	-5.2	0.59	<0.01	-9.0	-9.6	-10	-7.6	-6.6	-10.3	1.5	0.81	0.82	0.04
Asp	0.5	0.7	0.81	<0.01	1.9	2.3	0.8	1.2	1.1	0.1	0.5	0.68	0.06	0.32
Cys	-2.2	-0.6	0.50	0.64	-2.0	-2.4	-5.0	-5.0	-5.7	-1.7	1.6	0.49	0.15	0.28
Gln	-1.8	-4.3	0.25	0.18	1.2	34	4.5	19	-2.9	0.4	15	0.34	0.60	0.72
Glu	20	26	0.89	<0.01	40	45	35	42	32	39	2.8	0.22	0.02	0.85
Gly	-21	-22	0.43	<0.01	-63	-72	-53	-45	-53	-45	5.9	0.52	0.04	0.33
Pro	-1.9	-3.0	0.63	0.37	-2.5	-5.0	-5.6	-6.4	-4.8	-6.2	1.4	0.10	0.35	0.86
Ser	-13	-14	0.31	0.02	-17	-25	-24	-21	-26	-34	3.3	0.24	0.05	0.19
Tyr	-5.6	-6.9	0.94	0.09	-3.5	-4.6	-4.9	-5.1	-6.3	-7.2	1.3	0.52	0.17	0.93
Total AA	-68	-82	0.73	0.08	-125	-122	-145	-84	-149	-135	24	0.24	0.52	0.43

¹Net hepatic flux was calculated as hepatic blood plasma flow × hepatic concentration – (portal plasma flow × portal concentration + hepatic arterial blood plasma flow × arterial concentration).

²Fisher's LSD test for treatment effect on difference between prepartum and 4 DIM.

³Fisher's LSD test for prepartum to differ from 4 DIM.

⁴Standard error of the mean (n = 4; except for 4 DIM HMBi where n = 3).

⁵Fixed effects when prepartum observations are excluded from data set.

***P* ≤ 0.01 (difference between means within DIM).

the greatest quantity by the liver; however, hepatic Glu release decreased from 4 to 29 DIM ($P = 0.02$).

Net Splanchnic Fluxes

The net splanchnic fluxes of total EAA and all individual EAA except for His increased ($P < 0.01$ to $P = 0.03$; Table 6) from prepartum to 4 DIM. Postpartum, the EAA was released by splanchnic tissues in increasing amounts as lactation progressed ($P < 0.01$ to $P = 0.08$) except for His ($P = 0.43$) and Trp ($P = 0.35$), for which the flux did not change from 4 to 29 DIM in accordance with the patterns of the portal fluxes.

The net splanchnic fluxes of Asp, Glu, Pro, and Tyr increased ($P < 0.01$) from prepartum to 4 DIM. For Ala, a change ($P = 0.05$) was observed from net splanchnic release prepartum to net splanchnic uptake at 4 DIM. The net splanchnic uptake of Ala on 4 DIM was changed into net release by 15 and 29 DIM ($P = 0.02$). For Cys, the opposite occurred from d 4 to 15 postpartum where the net splanchnic release changed into net uptake ($P = 0.02$). For Gly, the prepartum to 4 DIM difference in net splanchnic uptake was affected by treatment ($P < 0.01$), reflected by a sharper increase in the net splanchnic uptake in HMBi compared with control. Glycine was the only AA for which there was net splanchnic uptake at all 3 postpartum sampling days, and a treatment by DIM interaction ($P < 0.01$) was observed, reflecting an initially larger net splanchnic uptake in HMBi compared with control (4 and 15 DIM) and similar values for both treatments at 29 DIM. The net splanchnic release of Pro was decreased ($P = 0.05$) in HMBi compared with control and increased ($P = 0.05$) for both treatments as lactation progressed.

Milk Recovery of EAA

The net portal and net splanchnic supply of EAA could not account for the EAA secreted in milk protein (Figure 1). Treatment did not affect ($P = 0.81$; Figure 1a) EAA deficiency relative to the net portal flux of EAA. The EAA balance relative to the net portal flux increased from -172 ± 24 g of EAA to $+10 \pm 22$ g of EAA between 4 and 29 DIM ($P < 0.01$; Figure 1a). The EAA balance relative to the net splanchnic flux of EAA increased from -248 ± 18 g of EAA to -74 ± 18 g of EAA between 4 and 29 DIM ($P < 0.01$; Figure 1b) and was not affected by treatment ($P = 0.33$). A total of $4,700 \pm 600$ g of EAA from extra-splanchnic sources were secreted in milk protein during the first 29 DIM.

DISCUSSION

Effects of HMBi Supplementation

Methionine has been identified as the most often limiting AA in dairy cows (Schwab et al., 1992). Supplementation with ruminally protected Met (Bach et al., 2000; Berthiaume et al., 2006) and the Met analog HMBi (Graulet et al., 2005; St-Pierre and Sylvester, 2005) have proven efficacious to improve the Met status of dairy cows by increasing Met concentrations in blood plasma. This was also observed in the present study, where supplementation with 1.5 g/kg DM of HMBi increased arterial Met in postpartum transition dairy cows within 4 d of dietary inclusion. Contrary to the response to the supplementation of ruminally protected Met (Bach et al., 2000; Berthiaume et al., 2006), supplementation with HMBi did not increase the net portal flux or net splanchnic flux of Met. On the contrary, we observed a tendency toward an increased hepatic uptake of Met, indicating an apparent decrease in net splanchnic output of Met with HMBi. These effects of HMBi on Met status and metabolism are in line with the previously observed conversion of HMB to Met in extra splanchnic tissues of ruminants (Lobley et al., 2006; Wester et al., 2006). Supplementation with HMBi at parturition immediately affected arterial Met and prevented the decrease in the arterial Met concentration observed in the nonsupplemented cows. However, the Met response did not persist, as the arterial concentration of Met in HMBi-supplemented cows decreased with time and did not differ from control cows at 29 DIM. This suggests an adaptation of tissue Met metabolism to increased Met supply.

In a continuous study design, St-Pierre and Sylvester (2005) observed an immediate milk production response to supplementation of 1.5 g of HMBi/kg of DM initiated at wk 4 of lactation, which persisted for the entire study of 16 wk. Several other studies have shown a response to HMBi on milk protein production (Phipps et al., 2008; Ordway et al., 2009). In the present study, the milk casein content in HMBi tended to decrease at a lower rate compared with control as lactation progressed. Previously, increased supply of metabolizable Met either from postruminal infusion (Pisulewski et al., 1996) or from HMBi supplementation (Rulquin et al., 2006) to cows in established lactation has increased the casein content without affecting the whey protein content of the milk.

A milk fat response to supplementary Met has been observed in numerous studies over a couple of decades (Polan et al., 1970; Emmanuel and Kennelly, 1984;

Table 6. Net splanchnic¹ fluxes of AA in periparturient Holstein cows supplemented with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi; 1.5 g/kg of DM) or not supplemented (CON) from the day of parturition

Plasma flux (mmol/h)	Prepartum		$P_{\text{trans} \times \text{trt}}$ ²	P_{trans} ³	4 DIM		15 DIM		29 DIM		SEM ⁴	P -value ⁵		
	CON	HMBi			CON	HMBi	CON	HMBi	CON	HMBi		Trt	DIM	Trt $\times$ DIM
Essential AA	55	73	0.92	<0.01	105	123	122	133	160	161	8.7	0.35	<0.01	0.79
Branched-chain AA	32	30	0.47	<0.01	63	68	70	74	90	89	4.0	0.68	<0.01	0.82
His	2.5	1.2	0.37	0.33	2.6	5.0	3.9	4.5	6.8	4.8	1.8	0.93	0.43	0.63
Ile	11	9.7	0.78	<0.01	19	19	19	22	26	26	1.6	0.39	<0.01	0.71
Leu	11	13	0.53	<0.01	25	29	28	30	34	36	1.6	0.10	<0.01	0.89
Lys	11	12	0.40	0.02	15	22	22	22	27	32	2.8	0.24	0.02	0.61
Met	1.9	1.7	0.52	<0.01	4.0	4.7	4.8	4.5	7.3	6.6	0.6	0.62	<0.01	0.63
Phe	2.2	2.3	0.71	<0.01	7.8	8.5	8.3	10	12	11	0.8	0.63	0.01	0.14
Thr	3.9	3.8	0.56	<0.01	10	12	11	16	14	16	1.4	0.10	0.08	0.37
Trp	0.7	0.5	0.92	0.03	2.0	1.7	1.6	2.4	2.8	2.6	0.5	0.89	0.35	0.52
Val	11	8	0.52	<0.01	20	20	23	22	30	27	1.9	0.45	<0.01	0.58
Nonessential AA	1.0	21	0.62	0.26	36	37	72	55	107	72	21	0.29	0.09	0.80
Ala	1.3	3.2	0.31	0.05	−3.3	−8.8	7.9	7.1	17	9.2	4.3	0.35	0.02	0.71
Asn	5.5	5.5	0.78	0.35	6.8	7.9	9.5	9.1	14	11	2.1	0.65	0.09	0.57
Asp	1.7	1.9	0.22	<0.01	4.6	4.3	3.4	3.5	4.6	3.6	0.7	0.62	0.02	0.21
Cys	0.6	1.0	0.63	0.21	1.7	2.0	−3.5	−1.5	−3.0	−1.1	1.5	0.32	0.02	0.94
Gln	−18	−8.8	0.59	0.34	−11	18	4.3	−2.7	31.5	−0.4	18	0.61	0.59	0.39
Glu	18	24	0.76	<0.01	42	47	38.7	44.6	37.4	44	2.6	0.16	0.23	0.96
Gly	−13	−12	<0.01	<0.01	−31	−51**	−8.8	−25**	−19	−18	3.5	0.06	<0.01	<0.01
Pro	2.4	2.6	0.28	<0.01	8.2	5.5	8.7	6.4	11.9	9.2	1.3	0.05	0.05	0.98
Ser	0.9	1.6	0.20	0.12	7.6	2.5	3.0	5.1	2.4	4.1	2.0	0.83	0.59	0.18
Tyr	1.4	1.5	0.61	<0.01	9.3	8.3	9.4	8.9	10	10	1.2	0.67	0.57	0.90
Total AA	55	73	0.92	<0.01	141	161	194	188	267	233	28	0.59	0.02	0.76

¹Net splanchnic flux was calculated as hepatic blood plasma flow $\times$ (hepatic concentration – arterial concentration).²Fisher's LSD test for treatment effect on difference between prepartum and 4 DIM.³Fisher's LSD test for prepartum to differ from 4 DIM.⁴Standard error of the mean (n = 4; except for 4 DIM HMBi where n = 3).⁵Fixed effects when prepartum observations are excluded from data set.** $P \leq 0.01$ (difference between means within DIM).

Figure 1. The deficiency in net portal flux (a) and net splanchnic flux (b) of essential AA (EAA) to account for milk secretion of EAA (g/d) in postpartum transition cows supplemented with the isopropyl ester of 2-hydroxy-4-(methylthio)-butanoic acid (Δ , HMBi) or unsupplemented (O, control) from the day of parturition. The deficiency relative to net portal flux was not affected by treatment ($P = 0.81$) and increased ($P < 0.01$) from -172 to $+10$ g/d of EAA between 4 and 29 DIM. The deficiency relative to net splanchnic flux was not affected by treatment ($P = 0.33$), and increased ($P < 0.01$) from -248 to -74 g/d of EAA between 4 and 29 DIM. Each data point is the mean of 4 observations $\pm$ SEM (except 4 DIM HMBi, where $n = 3$).

Varvikko et al., 1999). However, the metabolic explanation for this response has not been finally settled and it is unclear why the milk fat response is inconsistent both among studies and Met sources (Phipps et al., 2008; Ordway et al., 2009; Patton, 2010). Stimulation of liver export of lipoproteins by Met supply has often been discussed; however, the hepatic export of triacylglycerol in lipoproteins is very sparse in ruminants compared with, for example, rats (Grummer, 1993) and it seems unlikely that Met supplementation has any major effects on liver lipoprotein export in dairy cows (McCarthy et al., 1968; Bertics and Grummer, 1999; Piepenbrink et al., 2004). Substantial amounts of phosphatidyl choline are secreted in milk fat globule membranes, where it accounts for approximately 30% of phospholipids (Kanno, 1990) and Met supply could replenish the methyl pool of the cow. However, as substantial remethylation of homocysteine takes place (Lobley et al., 1996) and the carbon sources for this remethylation are readily available in form of Ser and Gly (perhaps also His) it could be speculated that the depletion of Met postpartum limits transmethylation reactions because too little Met is available for S-adenosylmethionine synthesis despite plentiful availability of methyl sources. Plasma levels of Ser and Gly are increased postpartum and the cows seem therefore not depleted in sources for methyl groups for remethylation, but arterial concentrations of Gly, Ser, and His decreased with HMBi supplementation, which could indicate a larger pull on these AA as methyl donors with an increased Met status. This is further supported by increased hepatic extraction rates of Ser

and His ($P < 0.01$ and $P = 0.07$; data not shown) postpartum in HMBi compared with control indicating an active hepatic response to decreasing arterial concentrations.

The net splanchnic flux of Leu tended to be greater for HMBi, which was brought about by decreased liver extraction. This agrees with observations by Berthiaume et al. (2006) where increasing doses of rumen-protected Met increased the splanchnic flux of Leu as well as the other branched-chain AA. Branched-chain AA are generally catabolized to a smaller extent by the liver compared with other EAA (Reynolds, 2006). Decarboxylation of the branched chain α -ketoacids by the branched chain α -ketoacid dehydrogenase complex is the rate-limiting step in the catabolic pathway and is shared by all the branched chain α -ketoacids but also the α -ketobutyrate derived from methionine (Bequette et al., 1998). Interaction between the substrates is a possible explanation as to why the greater removal of Met affects the hepatic metabolism of Leu with HMBi.

The tendency for reduced concentration of His as a response to HMBi supplementation agrees with the previously proposed mechanism (Schwab et al., 1992) that enhanced Met status improves the utilization of other EAA, although we cannot distinguish the use of His in protein synthesis and catabolic reactions including methylation reactions.

Overall, HMBi appear to be a fast-acting source of metabolizable Met also in postpartum transition dairy cows and HMBi supplementation prevented a decrease in the arterial Met concentration observed in unsupplemented cows.

AA Balance of the Postpartum Transition Cow

The transition from no milk protein production prepartum to 1.2 kg of protein/d within 4 d challenges the AA status of the cow. The EAA drainage by the mammary gland decreased the circulating levels of EAA in line with previous observations (Meijer et al., 1995; Larsen and Kristensen, 2009) on transition cows. Using indirect measures of changes in body composition, losses of body protein during early lactation are estimated to be 8 kg from 2 wk prepartum to 8 wk postpartum (Phillips et al., 2003) and 21 kg from 2 wk prepartum to 5 wk postpartum (Komaragiri and Erdman, 1997). In a serial slaughter trial, Gibb et al. (1992) estimated the net whole-body protein loss to be 5.6 kg during the first 8 wk of lactation.

It has been speculated that splanchnic tissues were responsible for the immediate supply of AA after parturition due to the high protein turnover in these tissues (Bell et al., 2000); however, the current study and the data from Larsen and Kristensen (2009) demonstrate that the crucial endogenous EAA supply were of extra-splanchnic origin. Also, previous protein depletion-repletion studies showed that skeletal muscles were by far the largest contributor to protein mobilization (Swick and Benevenga, 1977). Moreover, increasing abundance of mRNA coding for proteins in the ubiquitin-mediated proteolytic pathway in muscle biopsies from both postpartum transition cows and sows (Clowes et al., 2005; Chibisa et al., 2008) support that milk protein secretion in the initial phase of the lactation is largely sustained by protein mobilization from skeletal muscle. From the present data, a whole-body EAA mobilization can be estimated at $4,700 \pm 600$ g during the first 29 DIM as a stepwise area under the curve estimation from the net splanchnic deficiency (Figure 1b).

Even though mobilization of protein in skeletal muscle is the largest endogenous source of AA in the early postpartum period, the immediate postpartum uterine involution might also be a quantitatively important AA source during the first days of lactation. The available pool of CP has been estimated to be between 1,100 g (Gibb et al., 1992) and 1,300 g (Andrew et al., 1994). Assuming 45% EAA in uterine protein, the cow will have from 500 to 600 g of EAA available from the uterus, and uterine EAA might be especially important in the first days of lactation due to an expected hyperbolic pattern of uterine involution (Hanigan et al., 2009).

AA Sources for Mammary Protein Excretion

Milk protein content decreases rapidly after calving to a minimum at 5 to 10 wk in milk (Ng-Kwai-Hang et al., 1984), similar to the decrease from 4.2 to 3.0%

observed in the present study. The concentration of both whey and casein in milk followed the same pattern as the total protein concentration. Further, the casein, whey, and NPN fractions were estimated to constitute approximately 80, 16, and 3%, respectively, of total N in milk protein, and this distribution was unaffected by DIM. The caseins are exclusively synthesized from free AA in the mammary gland, whereas the whey fraction consists of both mammary-synthesized proteins (α -LA and β -LG) and proteins derived from the blood (e.g., immunoglobulins, serum albumin). The proteins derived from blood comprise a fraction of milk N not accounted for by the splanchnic release of EAA; however, the immunoglobulin levels in milk are expected to have been extensively reduced by 4 DIM (Levieux and Ollier, 1999; Madsen et al., 2004) and will therefore account for only a minor contribution to the 250 g of EAA in milk protein originating from endogenous sources. This corresponds to previous reported milk protein proportions (Cerbulis and Farrell, 1975) and demonstrates that the high milk protein concentration at 4 DIM is not only a result of immunoglobulin and plasma proteins transferred to the milk. The postpartum decrease in plasma protein content seems not to be related to a continuous transfer of quantitatively significant amounts of proteins or peptides from blood to milk. The present study does not allow for any assessment of an eventual net splanchnic flux of peptide-bound AA taken up by the mammary gland, but as this pathway seems to be of limited quantitative importance (Backwell et al., 1996), we infer that both AA available from dietary sources and the AA mobilized are transported to the mammary gland as free AA and not as intact proteins from plasma.

CONCLUSIONS

The hydroxy analog of methionine 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (HMBi) is a fast-acting source of metabolizable Met in postpartum transition dairy cows. When HMBi was supplemented from the day of parturition, the arterial blood plasma concentration of Met increased for the first 4 DIM and hepatic uptake of Met tended to increase during the entire period of supplementation. Supplementation with HMBi tended to increase milk fat content and decrease plasma concentrations of Ser, Gly, and His, pointing toward increased methyl-group transfer activity in response to HMBi supplementation and increased Met status. Cows mobilized substantial amounts of EAA from extra-splanchnic tissues to sustain milk protein production for the first 29 DIM, and mobilization was not affected by HMBi supplementation. The relative contents of casein and whey fractions in milk protein

were not affected by HMBi supplementation or DIM, and we infer that milk protein synthesis was sustained by free AA derived from uterine involution and mobilization from skeletal muscle.

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