



The effect of increasing concentrations of DL-methionine and 2-hydroxy-4-(methylthio) butanoic acid on hepatic genes controlling methionine regeneration and gluconeogenesis

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ABSTRACT

Metabolizable methionine (Met) concentrations can be increased by feeding rumen-protected DL-Met or the isopropyl ester of 2-hydroxy-4-(methylthio) butanoic acid (HMBi). Hepatic responses to increasing concentrations of metabolizable Met as a result of supplementation of different Met sources have not been comparatively examined. The objective of this experiment was to examine the regulation of key genes for Met metabolism, gluconeogenesis, and fatty acid oxidation in response to increasing concentrations of DL-Met or 2-hydroxy-4-(methylthio) butanoic acid (HMB) in bovine primary hepatocytes. Hepatocytes isolated from 4 Holstein calves less than 7 d old were maintained as monolayer cultures for 24 h before addition of treatments. Cells were then exposed to treatments of DL-Met or HMB (0, 10, 20, 40, or 60 μ M) in Met-free medium for 24 h and collected for RNA isolation and quantification of gene expression by quantitative PCR. Expression of betaine-homocysteine methyltransferase (*BHMT*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), and 5,10 methylenetetrahydrofolate reductase (*MTHFR*) genes, which catalyze regeneration of Met from betaine and homocysteine, decreased linearly with increasing DL-Met concentration. We observed similar effects with increasing HMB concentration, except expression of *MTHFR*, which was not altered. Expression of Met adenosyltransferase 1A (*MAT1A*), which catalyzes the first step of Met metabolism to generate S-adenosylmethionine (SAM), a primary methyl donor, was decreased with increasing DL-Met or HMB concentration. Expression of S-adenosylhomocysteine hydrolase (*SAHH*) was decreased linearly with increasing HMB concentration, but not altered by DL-Met. Increasing concentrations of DL-Met and HMB decreased cytosolic phosphoenolpyruvate

carboxykinase (*PCK1*) expression, but did not alter the expression of mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*) or pyruvate carboxylase (*PC*). Expression of glucose-6-phosphatase (*G6PC*) decreased linearly with increasing HMB concentration, but not altered by DL-Met. Neither DL-Met nor HMB altered the expression of carnitine palmitoyltransferase 1A (*CP-T1a*). These findings demonstrate reduced necessity for Met regeneration with increased Met concentrations in the medium, regardless of the Met source. The lack of upregulation of gluconeogenesis indicates that increased DL-Met or HMB is not prioritized for glucose synthesis in primary bovine hepatocytes.

Key words: 2-hydroxy-4-(methylthio) butanoic acid (HMB), methionine cycle, methyl donor, gluconeogenesis

INTRODUCTION

The importance of methionine (Met) is highlighted by its role as a limiting amino acid for protein synthesis in growing and lactating dairy cattle (Schwab et al., 1992; NRC, 2001). Different sources of Met—including rumen-protected Met and Met hydroxy analogs such as 2-hydroxy-4-(methylthio) butanoic acid (HMB) and the isopropyl ester of HMB (HMBi)—have been developed as dietary supplements to achieve adequate Met supply (Zanton et al., 2014). Increased milk protein concentration, milk yield, or both have been observed following supplementation with rumen-protected Met (Rulquin et al., 2006; Osorio et al., 2013) or HMBi (Ordway et al., 2009; Osorio et al., 2013). Because of rumen degradation, increased milk yield and milk protein concentration have not been observed following supplementation with HMB (St-Pierre and Sylvester, 2005; Rulquin et al., 2006).

In addition, Met serves as one of the dietary sources for methyl groups and is involved in methyl group metabolism; dietary methionine deficiency results in hepatic steatosis (Kharbanda, 2009). Methionine can be transformed into S-adenosyl methionine (SAM), the primary methyl donor, and SAM-dependent methyl-

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tion of phosphatidylethanolamine produces phosphatidylcholine, the major component of very low density lipoproteins (Purohit et al., 2007).

The liver plays a central role in Met metabolism (Finkelstein, 1990): approximately 50% of Met metabolism and up to 85% of all methylation reactions occur in the mammalian liver (Mato et al., 2002). Hepatic gene regulation serves as an important regulatory point for controlling Met metabolism (Finkelstein, 2003; Mato et al., 2008). S-Adenosyl-methionine synthetase (**MATI-III**) catalyzes the first step of the Met cycle to form SAM (Martinov et al., 2010). The removal of S-adenosyl-homocysteine (**SAH**) is catalyzed by the enzyme encoded by SAH hydrolase gene (*SAHH*), forming homocysteine and adenosine (Lu, 2000; Turner et al., 2000). Homocysteine is used by the transsulfuration pathway or enters the Met cycle to regenerate Met, which is catalyzed by 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), 5,10 methylenetetrahydrofolate reductase (*MTHFR*), and betaine-homocysteine methyltransferase (*BHMT*) (Finkelstein, 1990; Mato et al., 2008).

The effects of DL-Met or HMB on expression of the key genes for Met metabolism have not been well characterized in cattle. The primary objective of the present research was to determine the regulation of key genes for Met metabolism in response to increasing concentrations of DL-Met or HMB. Additionally, Met is a glucogenic amino acid, and gluconeogenesis is critical to support production of milk lactose, a major determinant of milk volume (Aschenbach et al., 2010). We were interested in testing whether the increased milk yield with Met or HMBi supplementation was due to increased gluconeogenic capacity, because substrate-induced gluconeogenesis has been reported previously (Zhang et al., 2015, 2016). The second objective was to evaluate the effects of DL-Met and HMB on expression of gluconeogenic genes, including cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*), pyruvate carboxylase (*PC*), and glucose-6-phosphatase (*G6PC*). We also examined expression of carnitine palmitoyltransferase 1A (*CPT1a*), a key gene for fatty acid transport, because Met is also involved in synthesis of carnitine (Mingrone, 2004), a required substance for transporting fatty acids from cytosol into mitochondria for β -oxidation (Bremer, 1983). Our hypothesis was that as concentrations of DL-Met or HMB increased, the need for cellular Met regeneration would decrease, and expression of key genes involved in the Met regeneration cycle would also decrease. In addition, as Met concentration increased, gluconeogenesis and fatty acid oxidation would be enhanced via upregulation of gluconeogenic genes and *CPT1a* expression.

MATERIALS AND METHODS

Hepatocyte Monolayer Preparation and Treatments

All experimental procedures involving animals were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. Four Holstein bull calves less than 7 d old (48 ± 2 kg of BW) were used as hepatocyte donor animals. Primary hepatocytes were isolated via collagenase perfusion of the caudate process as described previously (Donkin and Armentano, 1993). Approximately 2.0×10^6 cells were seeded on 35 mm Corning Primaria culture dishes (Fisher Scientific, Pittsburgh, PA), at a cell density of 2.0×10^5 cells/cm², with Dulbecco's modified Eagle medium (DMEM, 2902, Sigma, St. Louis, MO) containing 20% fetal bovine serum (Sigma) and 1% antibiotic, antimycotic solution (Sigma). Four hours after seeding, medium was aspirated and replaced with DMEM containing 10% fetal bovine serum and 1% antibiotic, antimycotic solution.

Twenty-four hours after seeding, cells were approximately 80% confluent, and medium was aspirated and replaced with Met-free DMEM containing 1% BSA (Merck Millipore, Billerica, MA) and 1% antibiotic, antimycotic solution, and plates were randomly assigned to treatments in triplicate. This low-glucose, Met-free DMEM contained 5.5 mM glucose and 1.0 mM sodium pyruvate, allowing us to establish an adequate experimental model to reflect the relevant physiological conditions of dairy cows in the periparturient state. Treatments included 0, 10, 20, 40, or 60 μ M DL-Met (Sigma) or HMB (Sigma). General cell health and morphology were monitored using the ZOE Fluorescent Cell Imager (Bio-Rad Laboratories Inc., Philadelphia, PA) throughout the experiment. After 24 h of treatment exposure, cells were collected in 0.5 mL of Trizol reagent (Life Technologies, Carlsbad, CA) and stored at -80°C until RNA analysis.

RNA Extraction, Real-Time RT-PCR, and Primer Evaluation

Total RNA was isolated using Trizol reagent. Samples were quantified and quality interrogated by absorbance using a Synergy hybrid spectrophotometer (BioTek, Winooski, VT). Each triplicate was pooled by equal quantity, and 100 μ L of the pooled RNA was further purified using the RNeasy Mini Kit (Qiagen Inc., Thousand Oaks, CA). A 0.5- μ g sample of purified RNA was reverse-transcribed to cDNA using iScript reverse transcriptase (Bio-Rad Laboratories Inc., Hercules, CA). Gene expression was quantified with real-time PCR using SsoFast EvaGreen supermix (Bio-

Rad Laboratories Inc.) and primers shown in Table 1. Primers were evaluated, and a single PCR product was verified using the following protocol: 1 cycle at 95°C for 3 min; 45 cycles at 95°C for 5 s, 55°C for 5 s; and a melt curve from 65°C to 95°C by 0.5°C increments for 3 s. A cDNA pool was generated using an equivalent quantity of cDNA from each sample. A 1:4 dilution series of the cDNA pool was used to generate the standard curve. A no-template control (water as a template) and no-reverse-transcription control (RNA pool as a template) were included in the real-time PCR analysis. All standards, controls, and samples were amplified in triplicate using the following reaction: 1 cycle at 95°C for 3 min; 45 cycles at 95°C for 5 s, 55°C for 5 s. The efficiencies of all PCR reactions were between 90 and 110% based on standard curve analysis. Quantification cycle data were transformed to starting quantity data using Bio-Rad CFX Manager software (version 3.1; Bio-Rad Laboratories Inc.) based on standard curves. The starting quantities for the 5 standards (1:4 dilutions of the cDNA pool) were 0.25, 0.0625, 0.0156, 0.0039, and 0.00098, respectively. The mRNA abundance of each target gene was the starting quantity of the target gene normalized to the arithmetic mean starting quantity of 3 reference genes: *18S*, ribosomal protein S9 (*RPS9*), and β -actin (*ACTB*) within each sample.

Statistical Analysis

All experiments were conducted in 4 separate cell preparations from 4 calves using 3 replicates per treatment. Data were analyzed using PROC MIXED in SAS 9.3 (SAS Institute Inc., Cary, NC). Two models of analyses of covariance were analyzed in response to Met source. The common slope model included the fixed effects of Met source and concentration, and the random effect of calf. The different slope model included the fixed effects of Met source, concentration, and source by concentration interaction, and the random effect of calf. The appropriate model for each gene was chosen based on the presence or absence of interaction, where no significant interaction indicated that the gene responded similarly to each source, and a significant interaction indicated that the gene responded differently to each source. Comparisons between Met sources at each concentration were made using the pdiff option. Data are reported as least squares means (**LSM**) and standard errors (**SE**). Statistical significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

We observed no effect of interaction between Met source and concentration on the mRNA expression of

Table 1. Primers used for target genes

Symbol	GenBank accession	Position ¹	Sequence (5'-3')
<i>18S</i>	NR_036642.1	F	ACCCATTGGAACGTCTGCCCTATT
		R	TCCTTGATGTGGTAGCCGTTTCT
<i>RPS9</i>	NM_001101152.2	F	CCTCGACCAAGAGCTGAAG
		R	CCTCCAGACCTCACGTTTGTTC
<i>ACTB</i>	NM_173979.3	F	GCGTGGCTACAGCTTCACC
		R	TTGATGTACACGGACGATTTTC
<i>MTR</i>	NM_001030298.1	F	GCCCCGTGAAAGGCAACAATG
		R	CATCCGGTAGGCCAAGTGTT
<i>BHMT</i>	NM_001011679.1	F	TGGGGGCAAAAATGTCAAGAAG
		R	TCCGTCTCCGATGATGACCT
<i>MTHFR</i>	NM_001011685.1	F	TGAGGGGAGACCCCATAGGT
		R	TCAGGTGCTTCAGATCAGCC
<i>MAT1A</i>	NM_001046497.1	F	GCCTGTGAGACGGTGTGTAA
		R	TAGCCAAACATCAGCCCCCTG
<i>SAHH</i>	NM_001034315.1	F	TCATTGAGACCCTCGTTGCC
		R	TCCAGGCGTACACTGGAATG
<i>PCK1</i>	NM_174737.2	F	AGGGAAATAGCAGGCTCCAGGAAA
		R	CACACGCATGTGCACACACACATA
<i>PCK2</i>	NM_001205594.1	F	TGACTGGGCAAGGGGAGCCG
		R	GGGGCCACCCCAAAGAAGCC
<i>PC</i>	NM_177946.4	F	CCACGAGT TCTCCAACACCT
		R	TTCTCCTCCAGCTCCTCGTA
<i>G6PC</i>	NM_001076124.2	F	TGATGGACCAAGAAAGATCCAGGC
		R	TATGGATTGACCTCACTGGCCCTCTT
<i>CPT1a</i>	NM_001304989.1	F	TTCGCGATGGACTTGCTGTA
		R	TTTCCTCCCGGTCCAGTTTG

¹F = forward primer; R = reverse primer.

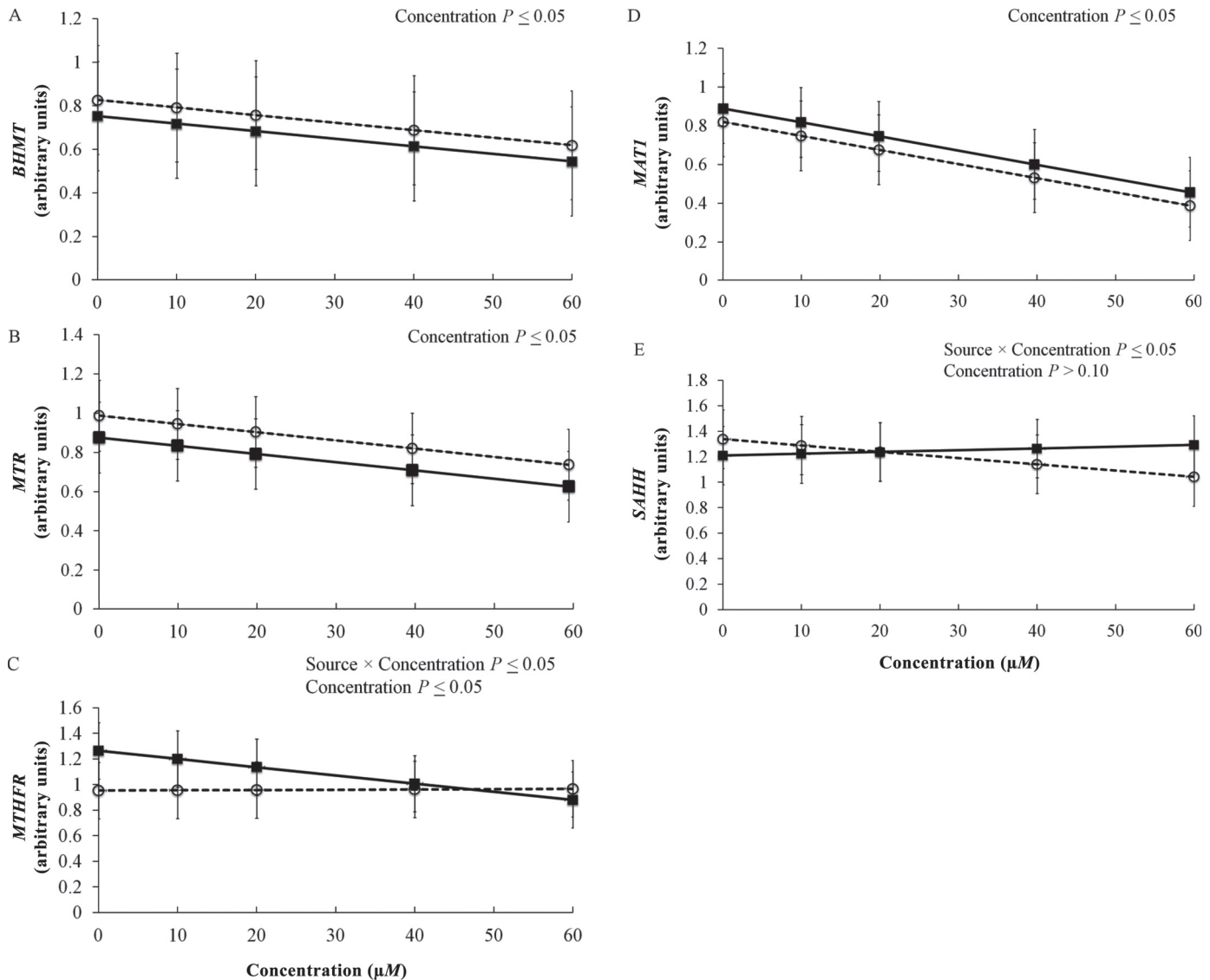


Figure 1. Effect of increasing concentrations of DL-methionine or 2-hydroxy-4-(methylthio) butanoic acid on expression of key enzymes involved in methionine metabolism in bovine calf hepatocytes. Cultured primary hepatocytes were exposed to 0, 10, 20, 40, or 60 μM of DL-methionine (■) or 2-hydroxy-4-(methylthio) butanoic acid (○) for 24 h and harvested for mRNA analysis. Data are expressed as arbitrary units of mRNA adjusted for the arithmetic mean abundance of 3 reference genes (*18S*, *RPS9*, and *ACTB*). Values are LSM and SE ($n = 4$ cell preparations). The interaction between methionine source and concentration was significant ($P \leq 0.05$) for *MTHFR* (5,10 methylenetetrahydrofolate reductase; panel C) and *SAHH* (S-adenosylhomocysteine hydrolase; panel E), but was not significant ($P > 0.10$) for *BHMT* (betaine-homocysteine methyltransferase; panel A), *MTR* (5-methyltetrahydrofolate-homocysteine methyltransferase; panel B), or *MAT1* (Met adenosyltransferase 1; panel D). The main effect of concentration was significant ($P \leq 0.05$) for *BHMT*, *MTR*, and *MAT1*. The main effect of methionine source was not significant ($P > 0.10$) for any gene.

BHMT, *MTR*, or *MAT1* ($P > 0.10$, Figure 1). We also observed no differences ($P > 0.10$) between DL-Met and HMB at any concentration tested for these 3 genes. Expression of *BHMT*, *MTR*, and *MAT1* mRNA decreased linearly ($P \leq 0.05$) in response to increasing Met concentrations, regardless of Met source (Figure 1).

We detected an interaction between Met source and concentration for *MTHFR* and *SAHH* mRNA ($P \leq$

0.05, Figure 1), but no differences ($P > 0.10$) between DL-Met and HMB at any concentration for *MTHFR* and *SAHH* mRNA. The interaction between Met source and concentration on *MTHFR* mRNA derived from the linear decrease ($P \leq 0.05$) and lack of change ($P > 0.10$) in response to increasing concentrations of DL-Met and HMB, respectively (Figure 1). In contrast, the interaction between Met source and concentration on

SAHH mRNA was due to the lack of change ($P > 0.10$) and linear decrease ($P \leq 0.05$) in response to increasing concentrations of DL-Met and HMB, respectively (Figure 1).

We observed no effect of interaction between Met source and concentration on the mRNA expression of *PCK1*, *PCK2*, *PC*, or *CPT1a* ($P > 0.10$, Figure 2). We also observed no differences ($P > 0.10$) between DL-Met and HMB at any concentration tested; instead, the interaction was explained by the lack of change ($P > 0.10$) and linear decrease ($P \leq 0.05$) of *G6PC* mRNA in response to increasing concentrations of DL-Met and HMB, respectively (Figure 2).

increasing DL-Met or HMB concentrations ($P \leq 0.05$, Figure 2). We detected no effects of Met concentration on *PCK2*, *PC*, or *CPT1a* mRNA ($P > 0.10$, Figure 2).

We detected an interaction between Met source and concentration for *G6PC* mRNA ($P \leq 0.05$, Figure 2). We observed no differences ($P > 0.10$) between DL-Met and HMB at any concentration tested; instead, the interaction was explained by the lack of change ($P > 0.10$) and linear decrease ($P \leq 0.05$) of *G6PC* mRNA in response to increasing concentrations of DL-Met and HMB, respectively (Figure 2).

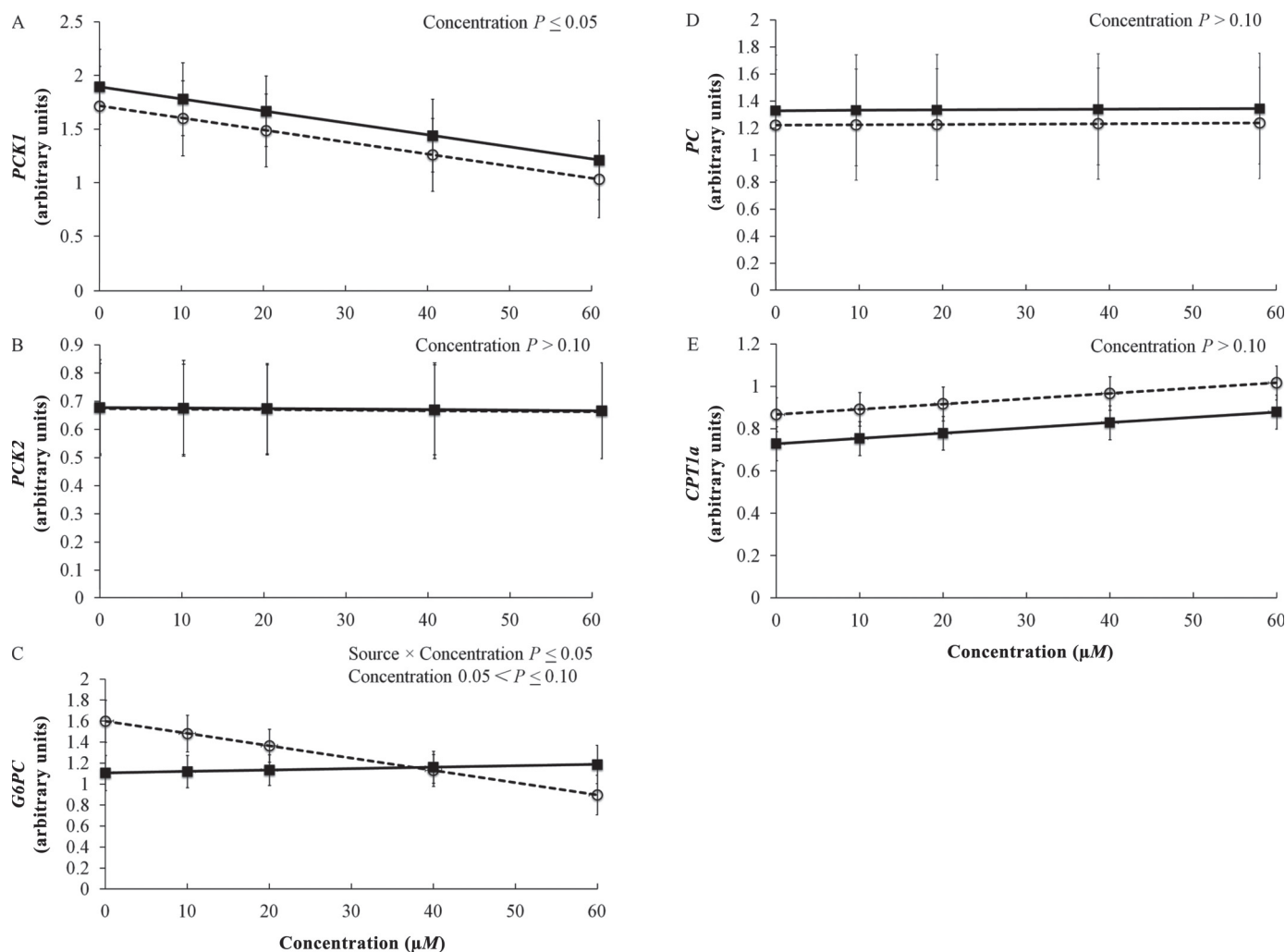


Figure 2. Effect of increasing concentrations of DL-methionine or 2-hydroxy-4-(methylthio) butanoic acid on expression of key enzymes involved in gluconeogenesis and fatty acid oxidation in bovine calf hepatocytes. Cultured primary hepatocytes were exposed to 0, 10, 20, 40, or 60 μM of DL-methionine (■) or 2-hydroxy-4-(methylthio) butanoic acid (○) for 24 h and harvested for mRNA analysis. Data are expressed as arbitrary units of mRNA adjusted for the arithmetic mean abundance of 3 reference genes (*18S*, *RPS9*, and *ACTB*). Values are LSM and SE ($n = 4$ cell preparations). The interaction between methionine source and concentration was significant ($P \leq 0.05$) for *G6PC* (glucose-6-phosphatase; panel C), and not significant ($P > 0.10$) for *PCK1* (cytosolic phosphoenolpyruvate carboxykinase; panel A), *PCK2* (mitochondrial phosphoenolpyruvate carboxykinase; panel B), *PC* (pyruvate carboxylase; panel D), or *CPT1a* (carnitine palmitoyltransferase 1A; panel E). The main effect of concentration was significant ($P \leq 0.05$) for *PCK1*, but not significant ($P > 0.10$) for *PCK2*, *PC*, or *CPT1a*. The main effect of methionine source was not significant ($P > 0.10$) for any gene.

DISCUSSION

The increased availability of Met in dairy cows has been associated with increased milk yield (Varvikko et al., 1999; Osorio et al., 2013), improved immunometabolic status (Osorio et al., 2014b), and modified hepatic methylation patterns (Osorio et al., 2016). The present study was designed to explore the direct role of increasing concentrations of DL-Met and HMB (2 Met sources available for liver metabolism in dairy cows) on hepatic mRNA expression of key genes for Met regeneration, gluconeogenesis, and fatty acids oxidation in hepatocytes cultured from preruminant calves. Primary hepatocyte cell culture is a useful model for studying underlying regulatory mechanisms, because the cultured cells maintain hormone, substrate, and pathway responsiveness (Donkin and Armentano, 1993, 1994; Donkin et al., 1997) while in vivo confounders (including differences in feed intake, nutrient status, and hormone concentrations) are reduced. However, it is important to ensure that cell culture conditions mimic physiological metabolite concentrations. The use of HMB instead of HMBi was to mimic the absorbed form of HMB that the liver is exposed to. The different effects of HMB and HMBi on lactation performance in vivo are due to their different sites of use. For example, HMB is highly degraded in the rumen, and only 5% of the ingested HMB enters the omasum; therefore, HMB is largely used in the rumen (Noftsker et al., 2005). Esterification of HMB to HMBi decreases the rate and extent of ruminal degradation of the HMB molecule (Robert et al., 2001; St-Pierre and Sylvester, 2005). Immediate appearance of HMB in peripheral blood after HMBi supply to the rumen indicates that HMBi is absorbed through the rumen wall and subsequently dissociates to yield HMB (Robert et al., 2001), and HMB is the form that is converted to L-Met in body tissues such as the liver (Robert et al., 2001; Lapierre et al., 2007, 2011). The liver extracts a considerable proportion (34 to 37%) of absorbed HMB and plays a critical role in the synthesis of Met from HMB in dairy cows (Lapierre et al., 2007, 2011).

The concentrations tested in the present study were chosen to mimic Met concentrations in cows fed diets deficient, adequate, or supplemented with Met. Arterial Met concentrations were 18 μM in cows that did not receive Met supplementation and were 45 μM in cows that received 72 g/d of rumen-protected Met during mid lactation (Berthiaume et al., 2001). Similarly, dietary supplementation of rumen-protected Met (0, 36, and 72 g/d) increased arterial Met concentrations (25, 29, and 40 μM) in early-lactation dairy cows (Berthiaume et al., 2006). Arterial Met concentration was reported to be 53.4 μM in mid-lactation cows that received jugu-

lar HMB infusion at a rate of 36 g/d (Lapierre et al., 2011). Arterial Met concentrations are readily available from feeding trials, but in hepatocyte cell culture experiments, treatments should mimic portal vein Met concentrations, which are what the liver is exposed to in vivo. Data on portal vein Met concentrations with dietary supplementation of rumen-protected Met or HMB are limited. One study indicated that portal vein Met concentrations were 49.8 μM in mid-lactation dairy cows that received an abomasal infusion of 15 g/d of DL-Met (Lapierre et al., 2012). The concentrations of 10 and 20 μM in the present study were chosen to mimic Met concentrations in cows fed Met-deficient diets, and 40 and 60 μM were chosen to mimic Met concentrations in cows fed Met-supplemented diets.

Met Regeneration

The first step of Met metabolism is catalyzed by MAT, an enzyme that has 3 distinct forms and is encoded by 2 different genes. The *MAT1A* gene is expressed exclusively in hepatic tissue and encodes MATI and MATIII; the *MAT2A* gene is expressed mainly in fetal hepatocytes and extrahepatic tissues and encodes MATII (Torres et al., 2000; Mato et al., 2002). Even though *MAT1A* expression decreased with increasing DL-Met and HMB concentrations, it did not necessarily reflect a reduction in SAM concentration. In fact, SAM concentration in the present study was likely to be maintained or elevated, knowing that SAM concentration is increased by rising methionine concentrations in mouse hepatocytes (Korendyaseva et al., 2008) and yeast cells (Sutter et al., 2013). Hepatic SAM was markedly elevated by excess dietary methionine in rats (Rowling et al., 2002), and both hepatic SAM and SAH concentrations increased by 4-fold when dietary methionine increased from 2 to 3% in rats (Finkelstein and Martin, 1986). Decreased *MAT1A* expression may be a regulatory mechanism for maintaining intracellular SAM concentration, which has been reported to be tightly controlled to a narrow range (Martinov et al., 2010) and considered a critical signal of amino acid sufficiency that reciprocally regulates cell growth and autophagy (Laxman et al., 2014). In addition, hepatic *MAT1A* expression is regulated mainly by DNA methylation and histone deacetylation (Mato et al., 2002). Transcription of the rat *MAT1A* gene is regulated by methylation of its promoter region, where a higher extent of methylation is related to lower mRNA expression (Torres et al., 2000). The potential increase in SAM in the present study may have enhanced the methylation of the *MAT1A* gene promoter, resulting in lower expression of *MAT1A*. These results are in contrast to another study, in which expression of *MAT1A*

mRNA in cultured rat hepatocytes was increased by 2 mM L-Met (Garcia-Trevijano et al., 2000); however, 2 mM is a supraphysiological concentration that was 33-fold higher than the highest Met dose used in the present study. Nonetheless, it is noteworthy that the enzyme activity of MAT is highly regulated posttranslationally (Mato et al., 2002), and even though they are encoded by the same *MAT1A* gene, the MATIII was activated by SAM in rats, but MATI activity was inhibited (Lu, 2000). The posttranslational regulation of MATI and MATIII, as well as the ratio between MATI and MATIII in response to Met and HMB supplementation in bovine hepatocytes, warrants further research.

The metabolite homocysteine is a major regulatory point for Met metabolism and can enter the transsulfuration pathway to generate products such as glutathione, or the Met cycle to regenerate Met. The enzymes encoded by betaine-homocysteine methyltransferase gene (*BHMT*) use betaine, an intermediate of choline metabolism, to regenerate Met from homocysteine (Finkelstein, 1990; Mato et al., 2008). When the dietary intake of labile methyl groups from methionine, betaine, and choline cannot meet the requirement of total consumption of methyl groups, the difference is made up via the methylneogenesis pathway through de novo synthesis of labile methyl groups, including conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate catalyzed by 5,10-methylenetetrahydrofolate reductase (*MTHFR*), and the subsequent transfer of the methyl group from 5-methyltetrahydrofolate to homocysteine via 5-methyltetrahydrofolate-homocysteine methyltransferase gene (*MTR*) (Finkelstein, 1990; Mato et al., 2008). Overall, the decreased mRNA expression of *MTR* and *BHMT* with increasing doses of either DL-Met or HMB, as well as the decreased *MTHFR* mRNA with increasing doses of DL-Met, may be a reflection of the reduced need to conserve Met. It has been suggested that homocysteine is preferably used via the transsulfuration pathway rather than Met regeneration when its concentration is elevated (Kharbanda, 2009). Increased glutathione production has been observed in the liver of dairy cows when Met is supplemented in the form of DL-Met or HMBi (Osorio et al., 2014b), which would support the shift in homocysteine metabolism.

Reduced *MTR* mRNA expression with increasing Met concentration in the present study is consistent with the previous observation that hepatic MTR activity was decreased in response to increased dietary Met supplementation in rats (Finkelstein and Martin, 1986) and steers (Lambert et al., 2002). In contrast to a previous whole-animal study, in which *BHMT* mRNA was not affected by dietary Met supplementation during the periparturient period in dairy cows (Osorio et al., 2014a), *BHMT* mRNA was reduced as the dose of Met increased

in the present study. Increased Met bioavailability is coupled with increased synthesis of SAM in the liver (Baldessarini, 1966; Finkelstein and Martin, 1986), and SAM serves as a negative regulator of BHMT activity (Finkelstein and Martin, 1984a,b). Moreover, the mRNA abundance of the *BHMT* gene is directly related to BHMT enzyme activity in rat liver (Park et al., 1997; Park and Garrow, 1999). Increased Met levels in the present study may have resulted in increased SAM concentrations, in turn downregulating the expression of this enzyme. A quadratic response of BHMT activity to increasing dietary Met supplementation in cattle (Lambert et al., 2002) and rats (Finkelstein et al., 1982) has been observed. The increased BHMT activity in response to either restricted or excessive Met was considered an important mechanism for conserving Met when Met supplementation was low, or for removing excessive homocysteine when Met supplementation was high (Finkelstein et al., 1982; Lambert et al., 2002). The absence of a quadratic response of *BHMT* mRNA in the present study may have been because even the highest Met concentration used was not excessive.

The enzyme encoded by the S-adenosyl-homocysteine hydrolase gene (*SAHH*) catalyzes the breakdown of SAH to homocysteine and adenosine (Turner et al., 2000). The SAM-dependent biological transmethylation is inhibited by its product SAH and a reduced SAM:SAH ratio. The decreased *SAHH* mRNA in response to increasing concentrations of HMB in the present study may result in elevated intracellular SAH levels, which in turn can affect expression of key gluconeogenic genes due to altered intracellular methylation processes (Jackson et al., 2012).

Gluconeogenesis and Fatty Acid Oxidation

Given the frequently observed increase in milk yield and the increase in milk protein with supplementation of rumen-protected Met (Rulquin et al., 2006; Osorio et al., 2013) or HMBi (Ordway et al., 2009; Osorio et al., 2013), as well as the critical role of gluconeogenesis in supporting the synthesis of milk lactose [the primary determinant for the milk volume (Aschenbach et al., 2010)], the second objective of the present study was to investigate the effects of Met and HMB supplementation on the gene expression of gluconeogenic enzymes in bovine hepatocytes. Methionine can serve as a gluconeogenic amino acid by entering the tricarboxylic acid cycle through succinyl-CoA, contributing to the mitochondrial oxaloacetate (**OAA**), a merging point for the entry of most gluconeogenic substrates to gluconeogenesis (Champe et al., 2008; Aschenbach et al., 2010). The enzyme encoded by *PC* catalyzes the formation of OAA from pyruvate, and the enzyme encoded by *PCK1* and

PCK2 catalyzes the formation of phosphoenolpyruvate from OAA in the cytosol and mitochondrion, respectively. It has been proposed that the entry of amino acids into gluconeogenesis is regulated by the enzyme encoded by *PC* and *PCK1*, rather than *PCK2*, because formation of phosphoenolpyruvate from amino acids requires independent synthesis of NADH in the cytosol (Aschenbach et al., 2010). The present data did not support our hypothesis that as Met concentration increased, excess Met would be used for gluconeogenesis through upregulation of the key gluconeogenic genes. The decreased *PCK1* and unchanged *PC* mRNA expression in response to increasing concentrations of DL-Met or HMB was also in contrast to an in vivo study, which found that supplemented DL-Met or HMBi increased hepatic *PCK1* mRNA and decreased *PC* mRNA (Osorio et al., 2016). One might argue that the discrepancy between this in vivo study and the present study in terms of *PCK1* and *PC* expression could be due to the insensitivity of hepatocytes isolated from preruminant calves to changes in gluconeogenesis. However, this was not likely to be the case knowing that *PCK1* and *PC* mRNA expression was increased in neonatal calf hepatocytes in response to nutritional stimuli such as the major gluconeogenic precursor propionate (Zhang et al., 2016). Instead, it is noteworthy that in the in vivo study, the responses of *PCK1* and *PC* mRNA to DL-Met or HMBi supplementation were observed only during the postpartum period, when feed intake was also increased, and not during the prepartum period, when feed intake was not altered (Osorio et al., 2016). Increased *PCK1* mRNA expression postpartum is due to increased feed intake (Greenfield et al., 2000; Agca et al., 2002), and therefore increased supply of propionate, which is a direct regulator of the *PCK1* promoter in dairy cows (Zhang et al., 2014 2015 2016). Conversely, upregulation of *PC* mRNA in vivo is observed at calving when feed intake is decreased or during intentional feed restriction (Greenfield et al., 2000; White et al., 2011). Therefore, the increased *PCK1* mRNA and decreased *PC* mRNA observed with Met supplementation previously (Osorio et al., 2016) were more likely to be a secondary effect of increased feed intake than a direct effect of DL-Met or HMB.

Because the mRNA abundance of *PCK1* and *PC* are directly related to their enzyme activities in dairy cows (Greenfield et al., 2000; Agca et al., 2002), the decreased *PCK1* mRNA and unchanged *PC* mRNA by increasing Met concentration should have resulted in reduced PCK-to-PC ratio, which may lead to increased intracellular OAA concentration. The size of the OAA pool affects the capacity of the tricarboxylic acid cycle to completely oxidize acetyl-CoA (Owen et al., 2002; White et al., 2011; White, 2015), so increased OAA

concentration may be beneficial for complete oxidation of acetyl-CoA generated from nonesterified fatty acids β -oxidation during negative energy balance in dairy cows.

The enzyme encoded by the *G6PC* gene catalyzes the last step of gluconeogenesis and glycogenolysis to release glucose (Nordlie and Foster, 2010). Our data were consistent with previous observations in rainbow trout hepatocytes, where expression of *G6PC* mRNA was depressed by L-Met (Lansard et al., 2011). Gluconeogenic genes, including *G6PC* and *PCK1*, are regulated at the transcriptional level by intracellular methylation processes regulating the activity of relevant key transcription factors (Jackson et al., 2012). Increased intracellular SAH concentration, and therefore decreased SAM:SAH ratio and impaired SAM-dependent protein methylation, resulted in decreased *G6PC* transcription in HepG2 human hepatocytes (Jackson et al., 2012). In the present study, the decreased *G6PC* mRNA with increasing concentration of HMB may have been due to decreased *SAHH* mRNA and presumably increased SAH concentration, as discussed above. In contrast to the consistent inhibition of *PCK1* mRNA by increasing Met concentration, expression of *G6PC* mRNA was differentially regulated by DL-Met and HMB. The lack of effects of DL-Met on *G6PC* and *SAHH* mRNA indicated that the inhibitory effect of Met on *G6PC* mRNA was specific to HMB.

It is important to note that even though Met can serve as a glucogenic amino acid, it is not prioritized for glucose production, and the quantitative contribution of essential amino acids to glucogenic carbon is less than 2.5% in transition dairy cows (Larsen and Kristensen, 2013). The fact that cows have metabolic priorities for Met other than hepatic gluconeogenesis has also been emphasized by a study in which abomasal infusion of free amino acids with casein profile, including Met, led to a greater true portal-drained viscera glucose release and increased milk lactose yield; however, hepatic true release of glucose was not affected in early-lactating dairy cows (Galindo et al., 2015). Given animals' many requirements for Met in a variety of fundamental biologic processes, including protein synthesis, protein start codon, SAM-dependent transmethylation reactions, formation of polyamines, synthesis of glutathione, and use as a precursor for the nonessential amino acid cysteine (Finkelstein, 1990), tightly regulated biological priorities for Met use that rate higher than gluconeogenesis are reasonable.

The protein encoded by *CPT1a* is a rate-limiting enzyme of hepatic mitochondrial fatty acid β -oxidation. The important role of Met in regulating *CPT1a* expression has been highlighted by a reduction of *CPT1a* mRNA accompanied by impaired hepatic fatty acid

oxidation and lipid accretion in rats (Serviddio et al., 2011) and chickens (Kikusato et al., 2015) fed a Met-deficient diet. Even though supplementation of Met in the form of DL-Met or HMBi in periparturient dairy cows increased hepatic concentration of free carnitine (Osorio et al., 2014b), a required substance for transporting fatty acids from cytosol into mitochondria for β -oxidation (Bremer, 1983), *CPT1a* mRNA was not altered in periparturient dairy cows fed rumen-protected Met (Osorio et al., 2016), consistent with the results of the present study. The nonresponsiveness of *CPT1a* mRNA expression to Met concentration may be specific to ruminant animals.

CONCLUSIONS

Decreased mRNA expression for genes that catalyze regeneration of Met (*BHMT*, *MTR*, and *MTHFR*) reflected the decreased need for cellular regeneration of Met with increasing Met concentrations. The subtle differences between Met sources on regulating expression of these genes indicated that HMB and DL-Met had similar sparing effects on Met regeneration in liver. Increasing concentrations of DL-Met and HMB did not upregulate key glucogenic genes; instead, expression of *PCK1* was decreased with increasing concentrations of DL-Met or HMB and *G6PC* was decreased with increasing concentration of HMB. These results indicate that increased Met supply in primary bovine hepatocytes is not directed at increased glucose synthesis, probably due to other metabolic priorities for Met. Further research is needed to elucidate the possible metabolic priorities for increased concentrations of Met in bovine hepatocytes.

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REFERENCES

- Agca, C., R. B. Greenfield, J. R. Hartwell, and S. S. Donkin. 2002. Cloning and characterization of bovine cytosolic and mitochondrial PEPCK during transition to lactation. *Physiol. Genomics* 11:53–63.
- Aschenbach, J. R., N. B. Kristensen, S. S. Donkin, H. M. Hammon, and G. B. Penner. 2010. Gluconeogenesis in dairy cows: The secret of making sweet milk from sour dough. *IUBMB Life* 62:869–877.
- Baldessarini, R. J. 1966. Alterations in tissue levels of S-adenosylmethionine. *Biochem. Pharmacol.* 15:741–748.
- Berthiaume, R., P. Dubreuil, M. Stevenson, B. W. McBride, and H. Lapierre. 2001. Intestinal disappearance and mesenteric and portal appearance of amino acids in dairy cows fed ruminally protected methionine. *J. Dairy Sci.* 84:194–203.
- Berthiaume, R., M. C. Thivierge, R. A. Patton, P. Dubreuil, M. Stevenson, B. W. McBride, and H. Lapierre. 2006. Effect of ruminally protected methionine on splanchnic metabolism of amino acids in lactating dairy cows. *J. Dairy Sci.* 89:1621–1634.
- Bremer, J. 1983. Carnitine-metabolism and functions. *Physiol. Rev.* 63:1420–1480.
- Champe, P. C., R. A. Harvey, and D. R. Ferrier. 2008. Amino acid degradation and synthesis. Pages 261–276 in Lippincott's Illustrated Reviews: Biochemistry. 4th ed. Lippincott Williams and Wilkins, Baltimore, MD.
- Donkin, S. S., and L. E. Armentano. 1993. Preparation of extended in vitro cultures of bovine hepatocytes that are hormonally responsive. *J. Anim. Sci.* 71:2218–2227.
- Donkin, S. S., and L. E. Armentano. 1994. Regulation of gluconeogenesis by insulin and glucagon in the neonatal bovine. *Am. J. Physiol.* 266:R1229–1237.
- Donkin, S. S., S. J. Bertics, and L. E. Armentano. 1997. Chronic and transitional regulation of gluconeogenesis and glyconeogenesis by insulin and glucagon in neonatal calf hepatocytes. *J. Anim. Sci.* 75:3082–3087.
- Finkelstein, J. D. 1990. Methionine metabolism in mammals. *J. Nutr. Biochem.* 1:228–237.
- Finkelstein, J. D. 2003. Methionine metabolism in liver diseases. *Am. J. Clin. Nutr.* 77:1094–1095.
- Finkelstein, J. D., B. J. Harris, J. J. Martin, and W. E. Kyle. 1982. Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine. *Biochem. Biophys. Res. Commun.* 108:344–348.
- Finkelstein, J. D., and J. J. Martin. 1984a. Inactivation of betaine-homocysteine methyltransferase by adenosylmethionine and adenosylethionine. *Biochem. Biophys. Res. Commun.* 118:14–19.
- Finkelstein, J. D., and J. J. Martin. 1984b. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. *J. Biol. Chem.* 259:9508–9513.
- Finkelstein, J. D., and J. J. Martin. 1986. Methionine metabolism in mammals. Adaptation to methionine excess. *J. Biol. Chem.* 261:1582–1587.
- Galindo, C., M. Larsen, D. R. Ouellet, G. Maxin, D. Pellerin, and H. Lapierre. 2015. Abomasal amino acid infusion in postpartum dairy cows: Effect on whole-body, splanchnic, and mammary glucose metabolism. *J. Dairy Sci.* 98:7962–7974.
- García-Trevijano, E. R., M. U. Latasa, M. V. Carretero, C. Berasain, J. M. Mato, and M. A. Avila. 2000. S-adenosylmethionine regulates MAT1A and MAT2A gene expression in cultured rat hepatocytes: A new role for S-adenosylmethionine in the maintenance of the differentiated status of the liver. *FASEB J.* 14:2511–2518.
- Greenfield, R. B., M. J. Cecava, and S. S. Donkin. 2000. Changes in mRNA expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation. *J. Dairy Sci.* 83:1228–1236.
- Jackson, M. I., J. Cao, H. Zeng, E. Uthus, and G. F. Combs. 2012. S-adenosylmethionine-dependent protein methylation is required for expression of selenoprotein P and gluconeogenic enzymes in HepG2 human hepatocytes. *J. Biol. Chem.* 287:36455–36464.
- Kharbanda, K. K. 2009. Alcoholic liver disease and methionine metabolism. *Semin. Liver Dis.* 29:155–165.
- Kikusato, M., S. Sudo, and M. Toyomizu. 2015. Methionine deficiency leads to hepatic fat accretion via impairment of fatty acid import by carnitine palmitoyltransferase I. *Br. Poult. Sci.* 56:225–231.
- Korendyaseva, T. K., D. N. Kuvатов, V. A. Volkov, M. V. Martinov, V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2008. An allosteric mechanism for switching between parallel tracks in mammalian sulfur metabolism. *PLOS Comput. Biol.* 4:e1000076.
- Lambert, B. D., E. C. Titgemeyer, G. L. Stokka, B. M. DeBey, and C. A. Löest. 2002. Methionine supply to growing steers affects hepatic activities of methionine synthase and betaine-homocysteine methyltransferase, but not cystathionine synthase. *J. Nutr.* 132:2004–2009.
- Lansard, M., S. Panerat, E. Plagnes-Juan, K. Dias, I. Seiliez, and S. Skiba-Cassy. 2011. L-leucine, L-methionine, and L-lysine are involved in the regulation of intermediary metabolism-related gene expression in rainbow trout hepatocytes. *J. Nutr.* 141:75–80.

- Lapierre, H., G. Holtrop, A. G. Calder, J. Renaud, and G. E. Lobley. 2012. Is d-methionine bioavailable to the dairy cow? *J. Dairy Sci.* 95:353–362.
- Lapierre, H., M. Vázquez-Añón, D. Parker, P. Dubreuil, G. Holtrop, and G. E. Lobley. 2011. Metabolism of 2-hydroxy-4-(methylthio) butanoate (HMTBA) in lactating dairy cows. *J. Dairy Sci.* 94:1526–1535.
- Lapierre, H., M. Vázquez-Añón, D. Parker, P. Dubreuil, and G. E. Lobley. 2007. Short communication: Absorption of 2-hydroxy-4-methylthiobutanoate in dairy cows. *J. Dairy Sci.* 90:2937–2940.
- Larsen, M., and N. B. Kristensen. 2013. Precursors for liver gluconeogenesis in periparturient dairy cows. *Animal* 7:1640–1650.
- Laxman, S., B. M. Sutter, and B. P. Tu. 2014. Methionine is a signal of amino acid sufficiency that inhibits autophagy through the methylation of PP2A. *Autophagy* 10:386–387.
- Lu, S. C. 2000. S-adenosylmethionine. *Int. J. Biochem. Cell Biol.* 32:391–395.
- Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. *Biochim. Biophys. Acta* 1804:89–96.
- Mato, J. M., F. J. Corrales, S. C. Lu, and M. A. Avila. 2002. S-Adenosylmethionine: A control switch that regulates liver function. *FASEB J.* 16:15–26.
- Mato, J. M., M. L. Martínez-Chantar, and S. C. Lu. 2008. Methionine metabolism and liver disease. *Annu. Rev. Nutr.* 28:273–293.
- Mingrone, G. 2004. Carnitine in type 2 diabetes. *Ann. N. Y. Acad. Sci.* 1033:99–107.
- National Research Council. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Noftsgger, S., N. R. St-Pierre, and J. T. Sylvester. 2005. Determination of rumen degradability and ruminal effects of three sources of methionine in lactating cows. *J. Dairy Sci.* 88:223–237.
- Nordlie, R. C., and J. D. Foster. 2010. A retrospective review of the roles of multifunctional glucose-6-phosphatase in blood glucose homeostasis: Genesis of the tuning/retuning hypothesis. *Life Sci.* 87:339–349.
- Ordway, R. S., S. E. Boucher, N. L. Whitehouse, C. G. Schwab, and B. K. Sloan. 2009. Effects of providing two forms of supplemental methionine to periparturient Holstein dairy cows on feed intake and lactational performance. *J. Dairy Sci.* 92:5154–5166.
- Osorio, J. S., C. B. Jacometo, Z. Zhou, D. Luchini, F. C. Cardoso, and J. J. Loor. 2016. Hepatic global DNA and peroxisome proliferator-activated receptor alpha promoter methylation are altered in periparturient dairy cows fed rumen-protected methionine. *J. Dairy Sci.* 99:234–244.
- Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. *J. Dairy Sci.* 96:6248–6263.
- Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2014a. Smartamine M and MetaSmart supplementation during the periparturient period alter hepatic expression of gene networks in 1-carbon metabolism, inflammation, oxidative stress, and the growth hormone–insulin-like growth factor 1 axis pathways. *J. Dairy Sci.* 97:7451–7464.
- Osorio, J. S., E. Trevisi, P. Ji, J. K. Drackley, D. Luchini, G. Bertoni, and J. J. Loor. 2014b. Biomarkers of inflammation, metabolism, and oxidative stress in blood, liver, and milk reveal a better immunometabolic status in periparturient cows supplemented with Smartamine M or MetaSmart. *J. Dairy Sci.* 97:7437–7450.
- Owen, O. E., S. C. Kalhan, and R. W. Hanson. 2002. The key role of anaplerosis and cataplerosis for citric acid cycle function. *J. Biol. Chem.* 277:30409–30412.
- Park, E. I., and T. A. Garrow. 1999. Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. *J. Biol. Chem.* 274:7816–7824.
- Park, E. I., M. S. Renduchintala, and T. A. Garrow. 1997. Diet-induced changes in hepatic betaine-homocysteine methyltransferase activity are mediated by changes in the steady-state level of its mRNA. *J. Nutr. Biochem.* 8:541–545.
- Purohit, V., M. F. Abdelmalek, S. Barve, N. J. Benevenga, C. H. Halsted, N. Kaplowitz, K. K. Kharbanda, Q. Liu, S. C. Lu, C. J. McClain, C. Swanson, and S. Zakhari. 2007. Role of S-adenosylmethionine, folate, and betaine in the treatment of alcoholic liver disease: summary of a symposium. *Am. J. Clin. Nutr.* 86:14–24.
- Robert, J. C., C. Richard, T. D'Alfonso, N. Ballet, and E. Depres. 2001. Investigation of the site of absorption and metabolism of a novel source of metabolizable methionine: 2-hydroxy-4-(methylthio) butanoic acid isopropyl ester (HMBi). *J. Dairy Sci.* 84(Suppl. 1):35. (Abstr.)
- Rowling, M. J., M. H. McMullen, D. C. Chipman, and K. L. Schalinske. 2002. Hepatic glycine N-methyltransferase is up-regulated by excess dietary methionine in rats. *J. Nutr.* 132:2545–2550.
- Rulquin, H., B. Graulet, L. Delaby, and J. C. Robert. 2006. Effect of different forms of methionine on lactational performance of dairy cows. *J. Dairy Sci.* 89:4387–4394.
- Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and V. M. Olson. 1992. Amino acid limitation and flow to the duodenum at four stages of lactation. 2. Extent of lysine limitation 1, 2. *J. Dairy Sci.* 75:3503–3518.
- Serviddio, G., A. M. Giudetti, F. Bellanti, P. Priore, T. Rollo, R. Tamborra, L. Siculella, G. Vendemiale, E. Altomare, and G. V. Gnoni. 2011. Oxidation of hepatic carnitine palmitoyl transferase-I (CPT-I) impairs fatty acid beta-oxidation in rats fed a methionine-choline deficient diet. *PLoS ONE* 6:e24084.
- St-Pierre, N. R., and J. T. Sylvester. 2005. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) and its isopropyl ester on milk production and composition by Holstein cows. *J. Dairy Sci.* 88:2487–2497.
- Sutter, B. M., X. Wu, S. Laxman, and B. P. Tu. 2013. Methionine inhibits autophagy and promotes growth by inducing the SAM-responsive methylation of PP2A. *Cell* 154:403–415.
- Torres, L., M. A. Avila, M. V. Carretero, M. U. Latasa, J. Caballería, G. López-Rodas, A. Boukaba, S. C. Lu, L. Franco, and J. M. Mato. 2000. Liver-specific methionine adenosyltransferase MAT1A gene expression is associated with a specific pattern of promoter methylation and histone acetylation: implications for MAT1A silencing during transformation. *FASEB J.* 14:95–102.
- Turner, M. A., X. Yang, D. Yin, K. Kuczera, R. T. Borchardt, and P. L. Howell. 2000. Structure and function of S-adenosylhomocysteine hydrolase. *Cell Biochem. Biophys.* 33:101–125.
- Varvikko, T., A. Vanhatalo, T. Jalava, and P. Huhtanen. 1999. Lactation and metabolic responses to graded abomasal doses of methionine and lysine in cows fed grass silage diets. *J. Dairy Sci.* 82:2659–2673.
- White, H. M. 2015. The role of TCA cycle anaplerosis in ketosis and fatty liver in periparturient dairy cows. *Animals (Basel)* 5:793–802.
- White, H. M., S. L. Koser, and S. S. Donkin. 2011. Bovine pyruvate carboxylase 5' untranslated region variant expression during transition to lactation and feed restriction in dairy cows. *J. Anim. Sci.* 89:1881–1892.
- Zanton, G. I., G. R. Bowman, M. Vázquez-Añón, and L. M. Rode. 2014. Meta-analysis of lactation performance in dairy cows receiving supplemental dietary methionine sources or postprandial infusion of methionine. *J. Dairy Sci.* 97:7085–7101.
- Zhang, Q., S. L. Koser, B. J. Bequette, and S. S. Donkin. 2015. Effect of propionate on mRNA expression of key genes for gluconeogenesis in liver of dairy cattle. *J. Dairy Sci.* 98:8698–8709.
- Zhang, Q., S. L. Koser, and S. S. Donkin. 2014. Propionate is a dominant inducer of bovine cytosolic phosphoenolpyruvate carboxykinase (PCK1) expression. *FASEB J.* 28:818–831.
- Zhang, Q., S. L. Koser, and S. S. Donkin. 2016. Propionate induces mRNA expression of gluconeogenic genes in bovine calf hepatocytes. *J. Dairy Sci.* 99:3908–3915.