Short communication: The effect of increasing concentrations of different methionine forms and 2-hydroxy-4-(methylthio)butanoic acid on genes controlling methionine metabolism in primary bovine neonatal hepatocytes

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ABSTRACT

The d-isomer of Met cannot be used directly by the mammary gland in dairy cows; instead, it is transformed into L-Met, the proteogenic isomer, in the liver and other extramammary tissues. It remains unclear whether different Met forms and a Met hydroxy analog, 2-hydroxy-4-(methylthio)butanoic acid (HMB), are metabolized and function similarly in the liver. The objective of the present study was to examine the regulation of key genes in Met regeneration, transulfuration, and transmethylation pathways in response to increasing doses of different Met forms. Hepatocytes isolated from 4 calves between 4 and 7 d old were maintained as monolayer cultures for 24 h before addition of treatments. Treatments of (0, 10, 20, 40 µM) d-Met, L-Met, dl-Met, dl-HMB, or a 1:1 mixture of dl-Met and dl-HMB were added to Met-free medium in triplicate. After 24 h, cell lysates were collected for quantification of gene expression by quantitative PCR, and mRNA abundance was normalized to the mean of 3 reference genes. Data were analyzed with PROC MIXED of SAS 9.3 (SAS Institute Inc., Cary, NC). Analyses of covariance confirmed equivalent slopes of Met form, and the final model included form, dose, and random effect of calf within form. Data are reported as least squares means ± standard error. No main effect of Met form was observed for any genes examined. The enzymes encoded by betaine-homocysteine methyltransferase (BHMT) and 5-methyltetrahydrofolate-homocysteine methyltransferase use betaine and 5-methyltetrahydrofolate, respectively, to regenerate Met from homocysteine. Increasing concentration of Met did not alter 5-methyltetrahydrofolate expression, but decreased BHMT expression. Expression of glycine N-methyltransferase, the enzyme that controls transmethylation flux from S-adenosyl-methionine, was not affected by Met concentration. Methionine concentration had no effect on expression of cystathionine β-synthase, a key enzyme for the transulfuration pathway. The decrease in BHMT expression indicates a decreased need for cellular Met regeneration with increasing Met concentration, independent of Met form. The lack of differences among Met forms on regulating genes examined indicates that all Met forms similarly reduced genes controlling Met regeneration and metabolism in primary bovine hepatocytes.

Key words: methionine isomer, methionine regeneration, 2-hydroxy-4-(methylthio)butanoic acid, transmethylation

Short Communication

Methionine is a limiting AA for protein synthesis in growing (Donahue et al., 1985) and lactating dairy cattle (Schwab et al., 1976, 1992; NRC, 2001). In addition to protein synthesis, Met serves multiple important biological roles (Figure 1). Utilization of D- and L-isomers of AA are AA- and species-dependent. In dairy cows, the D-Met isomer cannot be directly used by the mammary gland; instead, it is converted into L-Met, the proteogenic isomer, in liver and other extramammary tissues (Lapierre et al., 2012). Despite this need for conversion of D-Met to L-Met, supplementation of rumen-protected DL-Met or the isopropyl ester of 2-hydroxy-4-(methylthio)butanoic acid (HMBi) in dairy cows has been associated with improved milk production, milk protein concentrations, and nitrogen utilization (St-Pierre and Sylvester, 2005; Chen et al., 2011; Osorio et al., 2013), better immunometabolic status (Osorio et al., 2014a, 2016), and modified hepatic gene expression and DNA methylation pattern (Osorio et al., 2014a, 2016).

The biologically active component of HMBi is 2-hydroxy-4-(methylthio)butanoic acid (HMB). When supplemented in a nonesterified form, little HMB is absorbed in the rumen (St-Pierre and Sylvester, 2005; Zanton et al., 2014); however, the esterification process of HMB to various alcohols deceases ruminal degradation of the HMB molecule and results in
postruminal HMBi bioavailability of 40 to 58% (Robert et al., 2001a,b, 2002; Schwab et al., 2001; Graulet et al., 2005; St-Pierre and Sylvester, 2005). After supplemental HMBi is absorbed across the rumen wall, it is hydrolyzed into HMB and isopropyl alcohol, and subsequently converted to Met and acetone, respectively (Graulet et al., 2005).

The liver plays a central role in Met metabolism and key hepatic genes serve as important regulatory points for controlling Met metabolism (Finkelstein, 1990; Figure 1). A previous study indicated subtle differences between DL-Met and HMB on regulating expression of key genes for Met metabolism, gluconeogenesis, and fatty acid oxidation (Zhang et al., 2016). Although it is apparent that hepatic tissue can convert D-Met to L-Met, it remains unclear if different isoforms of Met differentially affect hepatic regulation of Met pathways, and whether interactions are present between DL-Met and HMB in liver. Our objective was to examine the regulation of key genes for transmethylation (methionine adenosyltransferase 1A, MAT1A; glycine N-methyltransferase, GNMT), Met regeneration (methionine reductase, MTR; betaine-homocysteine methyltransferase, BHMT), and transsulfuration (cystathionine-β-synthase, CBS; cystathionase, CSE) pathways in response to increasing concentrations of different Met sources, including D-Met, L-Met, DL-Met, HMB, and a 1:1 mixture of DL-Met and HMB in bovine hepatocytes. We hypothesized that different Met sources would be metabolized and function similarly in bovine hepatocytes and increased concentration of Met sources would decrease the expression of genes controlling methionine regeneration.

All experimental procedures involving animals were approved by the University of Wisconsin–Madison College of Agricultural and Life Sciences Animal Care and Use Committee. Four Holstein bull calves between 4 and 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 × 10⁶ cells were seeded on 35-mm Corning Primaria culture dishes (Fisher Scientific, Pittsburgh, PA) at cell density of 2.0 × 10⁵ cells/cm² (to obtain 80% confluence) with Dulbecco’s modified Eagle medium (DMEM 2902, Sigma, St. Louis, MO).

![Figure 1. Hepatic Met metabolism and the biological roles of Met. Betaine-homocysteine methyltransferase (BHMT), cystathionine-β-synthase (CBS), cystathionase (CSE), dimethylglycine (DMG), glutathione (GSH), glycine N-methyltransferase (GNMT), homocysteine (Hcy), methionine adenosyltransferase 1A (MAT1A), methionine reductase (MTR), methyltransferases (MT), 5-methyltetrahydrofolate (5-MTHF), S-adenosylhomocysteine (SAH), SAH hydrolase (SAHH), S-adenosylmethionine (SAM), and tetrahydrofolate (THF). Adapted from Chandler and White, 2017.](image-url)
containing 20% fetal bovine serum (Sigma) and 1% antibiotic, antimycotic solution (Sigma). Four hours after seeding, the medium was aspirated and replaced with DMEM containing 10% fetal bovine serum and 1% antibiotic, antimycotic solution.

Twenty-four hours after seeding, the medium was aspirated and replaced with Met-free DMEM containing 1% BSA (Merck Millipore, Billerica, MA) and 1% antibiotic, antimycotic solution. Plates were randomly assigned to treatments of 0, 10, 20, 40 µM of d-Met, L-Met, Dl-Met, HMB, or a 1:1 mixture of Dl-Met and HMB, which were applied in triplicate. After 24 h of treatment exposure, cell lysates were collected in 0.5 mL of Trizol reagent (Life Technologies, Carlsbad, CA) and stored at −80°C until subsequent RNA analysis.

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 µL of the pooled RNA was further purified using the RNAeasy Mini Kit (Qiagen Inc., Thousand Oaks, CA). A total of 0.5 µg of purified RNA sample were 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.) based on standard curves. The quantification cycle data were transformed to starting quantity (SQ) data with standard curve analysis. The quantification cycle data of all PCR reactions were between 90 and 110% based on the average efficiency of the primers. The primers were designed using Primer3 software.

The previous findings indicated that primary bovine fetal hepatic cells maintained a constant intracellular SAM concentration when Met concentrations, and random effect of calf. Data are reported as least squares means and standard errors. Statistical significance was declared at P < 0.05 and tendencies at 0.05 ≤ P ≤ 0.10.

Increasing concentrations of Met tended to decrease (P = 0.08) expression of MAT1A, which converts Met to S-adenosyl methionine (SAM), the primary methyl donor (Figure 2). Increasing concentrations of Met did not alter (P > 0.10) expression of MTR, but decreased (P < 0.05) BHMT, which uses 5-methyltetrahydrofolate and betaine, respectively, to regenerate Met. Decreases in MAT1A and BHMT in the current study are consistent with a previous, similar experiment that examined the effect of Dl-Met and HMB in primary bovine neonatal hepatocytes (Zhang et al., 2016). In the prior experiment, a decrease in MTR expression was observed, although the difference could have been due to the broader range of concentrations examined with a maximal dose of 60 µM. Decreased expression of genes associated with Met regeneration supports a need for Met regeneration with increasing Met supplementation. The decreased MAT1A expression may be a regulatory mechanism to maintain constant intracellular SAM concentration when Met concentration was increased.

Increasing concentrations of Met did not alter (P > 0.10) expression of GNMT, the major SAM-dependent transmethylase, or CBS and CSE, the 2 key enzymes for cysteine generation through the transsulfuration pathway (Figure 2). Although decreased expression of GNMT was observed previously during a LPS challenge in cell culture, CBS and CSE were not altered by the challenge (Zhang and White, 2017). Given that the current experiment did not involve an inflammatory challenge, it is not surprising that no changes were observed in expression of these genes.

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Figure 2. Effect of increasing concentrations of d-methionine (D), l-methionine (L), dl-methionine (DL), 2-hydroxy-4-(methylthio)butanoic acid (HMB), and a 1:1 mixture of DL and HMB (DL+HMB) on mRNA expression of the key enzymes including methionine adenosyltransferase 1A (A; MAT1A), glycine N-methyltransferase (B; GNMT), methionine reductase (C; MTR), betaine-homocysteine methyltransferase (D; BHMT), cystathionase (E; CSE), and cystathionine-β-synthase (F; CBS) relative to reference genes in bovine hepatocytes.
and Dl-HMB. In the current experiment, Met source had no effect (P > 0.10) on mRNA expression of any genes examined. This lack of differential response of primary bovine neonatal hepatocytes to different forms of Met, HMB, or a mixture supports that expression of key hepatic genes is similarly responsive to different Met forms.

The current work did not examine the rate of conversion of D-Met or HMB to L-Met, which has previously been suggested to be a slow process (Lapierre et al., 2012). A slow conversion of D-Met to L-Met could result in delayed availability to tissues or artificially increased extracellular concentrations; however, continual dietary supply in vivo should not present a confounder. Within the current experiment, all supplemental Met was provided at the beginning of the 24-h treatment period, which was apparently sufficient time for conversion of Met forms and alterations in gene expression within the cell culture system. Further elucidation of the conversion rate by hepatocytes in vitro would require time lapse treatment and sample collection.

In conclusion, Met supplementation reduces the necessity for Met regeneration and decreased MAT1A expression may be a means to maintain constant intracellular SAM concentration despite increased Met concentration. The lack of differences among Met sources on gene expression indicates that Met sources did not differentially regulate key genes involved in Met regeneration or metabolism pathways.

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REFERENCES


