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ORIGINAL CONTRIBUTION

Betaine is as effective as folate at re-synthesizing methionine for protein synthesis during moderate methionine deficiency in piglets

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Abstract
Purpose Both folate and betaine (synthesized from choline) are nutrients used to methylate homocysteine to reform the amino acid methionine following donation of its methyl group; however, it is unclear whether both remethylation pathways are of equal importance during the neonatal period when remethylation rates are high. Methionine is an indispensable amino acid that is in high demand in neonates not only for protein synthesis, but is also particularly important for transmethylation reactions, such as creatine and phosphatidylcholine synthesis. The objective of this study was to determine whether supplementation with folate, betaine, or a combination of both can equally re-synthesize methionine for protein synthesis when dietary methionine is limiting.
Methods Piglets were fed a low methionine diet devoid of folate, choline, and betaine, and on day 6, piglets were supplemented with either folate, betaine, or folate + betaine ($n = 6$ per treatment) until day 10. [1-^{13}C]-phenylalanine oxidation was measured as an indicator of methionine availability for protein synthesis both before and after 2 days of supplementation.
Results Prior to supplementation, piglets had lower concentrations of plasma folate, betaine, and choline compared to baseline with no change in homocysteine. Post-supplementation, phenylalanine oxidation levels were 20–46 % lower with any methyl donor supplementation ($P = 0.006$) with no difference among different supplementation groups. Furthermore, both methyl donors led to similarly lower concentrations of homocysteine following supplementation ($P < 0.05$).
Conclusions These data demonstrate an equal capacity for betaine and folate to remethylate methionine for protein synthesis, as indicated by lower phenylalanine oxidation.

Keywords Piglet · Folate · Betaine · Protein synthesis · 1-Carbon metabolism

Introduction

The amino acid methionine is important in the neonate not only for protein synthesis and growth, but once adenylated to *S*-adenosylmethionine (SAM) is also the universal methyl donor in over 50 transmethylation reactions [1]. The availability of methionine for its various functions is dependent on dietary methionine as well as the remethylation of methionine using folate or betaine (from choline), which can all be consumed in the diet. In the neonatal piglet, ~50 % of methionine is converted to SAM, while at 1 month old, only 30 % of methionine is utilized for transmethylation reactions [2]. Furthermore, the fraction of homocysteine remethylated to methionine by 5-methyltetrahydrofolate and betaine in the neonate is ~45 %, whereas only 20 % of homocysteine undergoes remethylation at 1 month old. This shows the significant demand placed on methionine for transmethylation reactions during the first weeks of life and demonstrates the importance of the remethylation nutrients, folate and betaine, to maintain methionine availability. Although the minimum dietary

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methionine requirement has been established in the human neonate, this value was determined under conditions of excess dietary cysteine [3] which has been shown in piglets to spare the methionine requirement by up to 40 % [4]. This sparing effect emphasizes that the methionine requirement is not static, and nutrients involved in both methionine demand (i.e., cysteine) and supply (i.e., folate, betaine) should be considered when determining the dietary methionine requirement.

SAM is utilized to expand body pools of transmethylation products, such as phosphatidylcholine (PC) and creatine, which increase in the neonatal piglet by ~10 % per day [5]. In addition, SAM provides the methyl group used to establish and maintain epigenetic patterns of DNA methylation, which may be permanently affected by the neonatal diet [6]. The methionine required for these reactions is significant considering a healthy neonate consuming 14 μmol kg^{-1} h^{-1} of methionine has a transmethylation rate of ~20 μmol kg^{-1} h^{-1} [7]. This high demand for transmethylation reactions highlights the importance in understanding how the dietary supply of methionine and methyl groups affects the availability of methionine for protein synthesis and growth in the neonate.

During transmethylation, SAM is converted to *S*-adenosylhomocysteine (SAH) which is further hydrolyzed to form homocysteine, a nonprotein amino acid that can either be irreversibly converted to cysteine or remethylated to methionine by receiving a methyl group from 5-methyltetrahydrofolate or betaine, the oxidation product of choline [8]. 5-Methyltetrahydrofolate provides a methyl group via the ubiquitous enzyme methionine synthase (MS), while betaine–homocysteine methyltransferase (BHMT) transfers a methyl group from betaine, a reaction that is limited to the liver and kidney [9]. Remethylation via betaine also produces dimethylglycine (DMG), an inhibitor of BHMT [10], which is oxidized to sarcosine via DMG dehydrogenase using folate as a cofactor [11].

Methionine availability will depend on dietary levels of these methyl-related nutrients. For the growing neonate, the concentrations of these nutrients are highly variable among infant formulas and breast milk. Serum-free choline is higher in breast-fed compared to formula-fed infants, and the choline concentration of breast milk is influenced by maternal choline status [12]. Although the average choline concentration in breast milk is ~1200 nmol ml^{-1}, the concentration of betaine is only 7 nmol ml^{-1} [13] making choline the primary source of betaine in the neonate. Moreover, most infant formulas are derived from cow's milk which has a higher casein/whey ratio than human breast milk. Because casein is high in methionine while whey is high in cysteine, cow's milk also has a higher methionine/cysteine ratio of ~3 versus ~0.8 found in human milk [14]. Other studies investigating the relationship between remethylation nutrients have demonstrated that dietary choline deficiency leads to lower hepatic folate [15], while folate deficiency results in hepatic depletion of choline [16], further emphasizing the need to assess the relationship between nutrients involved in methionine metabolism when considering individual nutrient requirements.

Because of the high demand placed on methionine during early development and the variability of methyl donors in the neonatal diet, the objective of this study was to determine how important remethylation is to methionine availability. Moreover, we wanted to compare the effectiveness of folate, betaine, or a combination of both to remethylate methionine in vivo. In order to elicit a response with remethylation, we employed a diet that was moderately deficient in methionine and used the indicator amino acid oxidation (IAAO) technique to detect changes in protein synthesis. The IAAO technique is based on the concept that when one amino acid is limiting for protein synthesis, the remaining amino acids will be oxidized; by increasing the availability of the limiting amino acid (i.e., more methionine from remethylation), the oxidation of other amino acids will decrease, reflecting a higher incorporation into protein [17]. Using phenylalanine as our indicator amino acid, we hypothesized that independently and in combination, methyl donor supplementation will rescue methionine deficiency as indicated by lower oxidation of [1-^{13}C]-phenylalanine.

Materials and methods

Animal protocol

All animal-handling procedures were approved by the institutional animal care committee in accordance with the guidelines of the Canadian Council on Animal Care (protocol #12-61-RB). Eighteen 5- to 8-day-old Yucatan miniature piglets were obtained from the Memorial University of Newfoundland breeding colony. Upon arrival, general anesthesia was induced with isoflurane (1–2 %) in oxygen (1.5 l min^{-1}), and piglets were implanted with two silastic venous catheters (femoral and jugular) for blood sampling and a gastric catheter for intragastric (IG) feeding following recovery [18]. Piglets were administered antibiotics via both venous catheters (Borgal: 20 mg trimethoprim and 100 mg sulfadoxine) (Intervet Canada Ltd.) immediately following surgery and daily for the duration of the study. Analgesic (0.03 mg kg^{-1} of buprenorphine hydrochloride) (Schering-Plough) was administered immediately following surgery as well as every 12 h post-surgery until no guarding was observed by piglets. Piglets were transferred to individual metabolic cages (1 m × 1 m) equipped with heat lamps in a room maintained at 28 °C and lit from 0800

to 2000 hours. Diet was administered using a peristaltic pump attached via a dual port swivel and tether system (Lomir Biomedical) that allowed for free movement of piglets around the cage while allowing for continuous IV or IG infusions. Blood was sampled daily using sodium heparin vacutainers, and plasma was separated and stored at −20 °C for future analyses. Piglets were weighed daily, and diet infusion rates were adjusted accordingly.

Diet regimen

Immediately following surgery (day 0), piglets were parenterally fed at 50 % of requirement via the jugular catheter. On the morning of day 1, diet delivery rate was increased to 75 % of the requirement, and by evening of the same day, diet delivery was changed to enteral feeding via the IG catheter at 100 % of target rate. From day 0 to day 5, piglets were fed an adaptation diet which provided adequate methionine (0.3 g kg^{-1} day^{-1}) (Fig. 1) [19] and was devoid of folate, betaine, and choline. On day 5, methionine was reduced to a moderately deficient level of 0.2 g kg^{-1} day^{-1} for the remainder of the 10-day study, and alanine was adjusted to maintain an isonitrogenous diet. On day 7, piglets were randomly assigned to receive supplementation with either folate ($n = 6$), betaine ($n = 6$), or folate + betaine ($n = 6$). Folate was supplemented at a rate of 38 µg kg^{-1} day^{-1}, providing 200 % of the daily folate requirement in a piglet (NRC 1998). Betaine was provided at a rate of 235 mg kg^{-1} day^{-1}; there is currently no specified requirement for betaine in piglets, and the dose provided in this study is the molar equivalent of the methionine requirement of 0.3 g kg^{-1} day^{-1}. Although the molar betaine supplementation level used in this study was higher than folate, betaine can only be synthesized via choline oxidation and cannot be re-synthesized following transfer of its methyl group, while the methyl group provided by folate can be regenerated via a variety of sources [20].

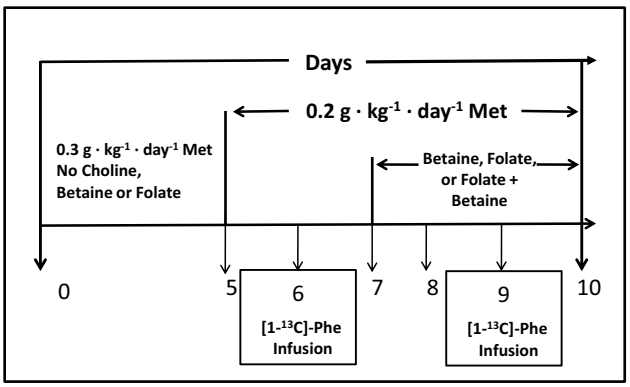

Fig. 1 Diet and infusion protocol. *Met* methionine, *Phe* phenylalanine

Both adaptation and test diets provided 16 g kg^{-1} day^{-1} of protein supplied by crystalline amino acids as previously described [18]. Nonprotein energy was provided in a 1:1 ratio of carbohydrate in the form of dextrose and fat provided as soybean oil which was administered into the diet line via a syringe pump at a rate of 0.12 ml kg^{-1} h^{-1}. Vitamins (Multi-12K Pediatric, Sabex) and minerals were provided in the diet at >100 % of the requirement, and diet was administered at a rate of 11.3 ml kg^{-1} h^{-1}. Diet was made under aseptic conditions as previously described [18].

Isotope infusion protocol

On days 6 and 9 of the study, piglets were moved to individual and sealed Plexiglas boxes (60 × 40 × 40 cm) fitted with a tether and swivel system to allow for blood collection via venous catheters, delivery of isotope, and continuous delivery of diet. A primed (7 mg kg^{-1}), constant (2.4 mg kg^{-1} administered every 30 min) infusion of L-[1-^{13}C]-phenylalanine (99 %, Cambridge Isotope Laboratories) was given intragastrically via the diet line for 6 h [21]. Blood was sampled every 30 min, and heparinized plasma was separated and stored at −20 °C for later analysis of [1-^{13}C]-phenylalanine enrichment. Breath samples were collected, and the rate of CO_2 production was determined via an indirect calorimetry system (Qubit Systems). A vacuum pump was used to draw air from the boxes at a fixed known rate controlled by a precision gas flow controller (Qubit Systems). Individual 20-min breath samples were collected using a 4-channel gas switcher to direct flow from a specified box into a midget bubbler (Kimble Chase) containing sodium hydroxide (1 M). Following the collection, the sodium hydroxide was quickly poured into an empty glass vacutainer, and the seal was immediately replaced. A syringe was used to evacuate air from the vacutainer which was then stored at 4 °C until further analysis for $^{13}CO_2$ enrichment. Following collection of breath, the channel gas switcher was used to direct air toward a CO_2 analyzer (Model CD-3A:AEI Technologies) to determine the concentration of CO_2 in the box and CO_2 production rate via the calorimetry system software (C960 multichannel gas exchange system software: Qubit Systems).

Plasma metabolites

Total homocysteine and cysteine concentrations were determined according to Vester [22]. Methionine concentrations were measured using phenylisothiocyanate derivatization and HPLC [23]. Concentrations of choline, DMG, and betaine were quantified using HPLC MS/MS as described elsewhere [24, 25]. Briefly, plasma samples were mixed with three volumes of acetonitrile containing 100 µM of D_{11}-betaine and D_9-choline. The supernatant was extracted,

and an autosampler was used to inject 1 µl onto an Atlantis HILIC Silica 3 µm, 2.1 × 100 mm column. Samples were analyzed on a Waters Alliance 2795 HPLC system (Waters Corporation) using a Micromass Quattro Ultima tandem mass spectrometer (Waters Corporation) using an isocratic buffer system of ammonium formate (15 mM, pH 3.5; 17.5 %) and acetonitrile (82.5 %) with a run time of 6 min at a flow rate of 0.6 ml min^{-1}. The column effluent was split at a ratio of 1:4 and delivered to the mass spectrometer at a rate of 150 µl min^{-1}. The compounds were detected in multiple-reaction monitoring mode using the following *m*/*z* transitions: D_{11}-Betaine 129 → 68, Betaine 118 → 59, D_9-Choline 113 → 69, Choline 104 → 60. Plasma concentrations were calculated using dialyzed plasma spiked with choline and betaine standards, and expressed relative to D_{11}-betaine and D_9-choline. Concentrations were computed using MassLynx Software (Waters Corporation). Plasma folate concentrations were determined using a folate kit as per manufacturer's instructions automated on an Architect i2000 immunoassay analyzer (B1P740: Abbott Diagnostics).

CO_2 enrichment in breath

Breath CO_2 was analyzed for ^{13}C isotope enrichment by isotope ratio mass spectrometry—Delta XP isotope ratio mass spectrometer interfaced to a Gas Bench II (Thermo Fisher Scientific). Briefly, 200 µl of the NaOH solution containing the sequestered CO_2 was transferred to Exetainers (Labco Limited) and capped. Using a 27-gauge needle and syringe, 300 µl of 1 M HCl was added to each Exetainer through the airtight septum. Samples were vortexed and allowed to stand at room temperature for 30 min before analysis by isotope ratio mass spectrometry. Mean $^{13}CO_2$ enrichment was compared against standardized reference gas which was calibrated against Vienna Pee Dee Belemnite.

Plasma enrichment of [1-^{13}C]-phenylalanine

The plasma enrichment of [^{13}C]-phenylalanine was analyzed using a modified gas chromatography–mass spectrometry (GCMS) method of Lamarre [26]. 50 µl of plasma was mixed with 135 µl of 133 mM pentafluorobenzyl bromide and 25 µl of 0.5 M phosphate buffer (pH 8) in an Eppendorf tube. Samples were placed into a 60 °C oven for 60 min to allow for alkylation of phenylalanine by pentafluorobenzyl bromide. 335 µl of hexane was added to terminate derivatization. After a second mixing, the organic phase was transferred to a GCMS vial. Derivatized samples were analyzed using a GCMS equipped with an autosampler (Agilent Technologies). A 2-µl aliquot of the organic phase was injected onto a DM-5MS column (0.25 mm × 30 m × 0.22 µm). The GC (6890 N Network GC System) oven was preheated to 50 °C and equipped with helium carrier gas. A temperature of 50 °C was held for 3 min and was increased to 280 °C at a rate of 30 °C min^{-1} which was then held for 4 min. After column separation, 20 eV of collision energy was used to ionize samples upon entering a quadrupole MS (5973 inert Mass Selective Detector). Selected-ion monitoring mode was used to monitor specific ions for phenylalanine ($m/z = 434$ and 435). Area under the curve for each ion was recorded and used to calculate the ratio of [M + 1] phenylalanine as compared to [M + 0] phenylalanine.

Calculations

Phenylalanine flux, rate of phenylalanine oxidation, and percent phenylalanine oxidized were calculated according to equations by Zello [27]:

Phenylalanine flux (Q): $Q = i[(E_i/E_p) - 1]$;

where E_i is the enrichment of [1-^{13}C]-phenylalanine infused (MPE), E_p is the enrichment of plasma [1-^{13}C]-phenylalanine above baseline at isotopic plateau (MPE), and i is the rate of [1-^{13}C]-phenylalanine infused (µmol kg^{-1} h^{-1}).

The rate of phenylalanine oxidation (Phe_{ox}):

$$Phe_{ox} = \left[(FCO_2 \times ECO_2 \times 44.6 \times 60)/(W \times 0.93 \times 100)\right] \times (1/E_p - 1/E_i) \times 100;$$

where FCO_2 is the CO_2 production rate (cm^3 min^{-1}) determined by Qubit Systems software as described above, ECO_2 is the $^{13}CO_2$ enrichment in expired breath at isotopic steady state (atom percent excess) determined by isotope ratio mass spectrometry, and W is the weight (kg) of the piglet. The constants 44.6 pmol cm^{-3} and 60 min h^{-1} converted FCO_2 to micromoles per hour, and the factor 100 changed atom percent excess to a fraction. 0.93 was used to account for CO_2 retention in piglets [28].

The %dose of [1-^{13}C]-phenylalanine oxidized (%Phe_{ox}) :

$$\%Phe_{ox} = [(FCO_2 \times ECO_2 \times 44.6 \times 60)/(W \times 0.93 \times 100)]/ i \times 100\,\%$$

Non-oxidative disposal (NOD) and phenylalanine released from protein (PB) were calculated according to House et al. [29] based on the following equation:

$$Q = NOD + Phe_{ox} = PB + I,$$

where Q is the phenylalanine flux as described above, NOD is the non-oxidative disposal of phenylalanine, Phe_{ox} is the phenylalanine oxidation, PB is the phenylalanine released from protein, and I is the phenylalanine intake. Using our

calculated phenylalanine flux and oxidation values, we were able to solve for NOD and PB.

Statistics

Data are presented as mean ± SD. The effect of methyl donor supplementation was assessed using a two-way ANOVA to compare groups; the two main effects were treatment (i.e., various methyl donors) and rescue (i.e., pre- and post-supplementation). Bonferroni posttests were used when main effects were identified by two-way ANOVA. Differences were considered significant at $P < 0.05$ (GraphPad Prism 4.0 for Windows, GraphPad Software).

Results

Piglet weight

Pre-rescue piglet weights were 2.4 ± 0.3, 2.4 ± 0.4, and 2.4 ± 0.2 kg for folate, betaine, and folate + betaine, respectively, and were higher post-rescue (2.9 ± 0.1, 2.9 ± 0.4, and 2.7 ± 0.3 kg for folate, betaine, and folate + betaine, respectively), with no differences in weight among supplementation groups.

Plasma metabolites following methyl deficiency

Because all piglets on day 7 were receiving the same diet, these data include all pigs. As a baseline reference, plasma concentrations of folate, betaine, and choline were measured in a separate group of 5- to 8-day-old sow-fed piglets ($n = 14$) and were compared to day 7 piglets from this study (12–15 days old). On day 7, plasma folate (16.1 ± 7.8 ng ml^{-1}) and choline (6.9 ± 6.8 μM) concentrations were lower than the day 0 reference group (folate: 45.7 ± 22.3 ng ml^{-1}; choline: 23.2 ± 14.1 μM). In all animals on day 7, plasma betaine concentration was below the limit of detection of the assay (<0.55 μM), compared to the betaine concentration of 48.7 ± 27.1 μM in the "day 0" reference group. Plasma homocysteine concentration on day 7 (30.0 ± 14.5 μM) was similar to reference group (28.8 ± 13.6 μM), whereas plasma methionine concentration on day 7 (11.4 ± 3.5 μM) was ~ 30 % lower than the reference group (16.0 ± 3.5 μM), reflecting the reduction in dietary methionine on day 5.

Plasma metabolites following supplementation

There was a main effect of rescue on plasma DMG concentration ($P = 0.017$) with ~75-fold higher DMG in both betaine-supplemented groups and ~45 % lower plasma DMG following folate supplementation alone, with no treatment × rescue interaction (Table 1). There was a treatment × rescue interaction in plasma methionine concentration with a lower concentration of methionine in betaine-supplemented piglets post-rescue (Table 1). There was a main effect of rescue on plasma homocysteine concentration with 12–65 % lower levels post-rescue versus pre-rescue ($P = 0.0009$) with no treatment × rescue interaction. There were no differences in choline or cysteine concentrations (Table 1). Folate rescue was confirmed with plasma folate changing from 14.6 ± 9.5 and 18.4 ± 3.1 ng ml^{-1} in folate, and folate + betaine groups, respectively, to above the analytical measurement range of the assay (>80 ng ml^{-1}) post-supplementation. Betaine-supplemented animals had plasma folate levels of 15.3 ± 10.4 and 38.8 ± 24.5 ng ml^{-1} pre- and post-supplementation, respectively. Plasma betaine concentration remained below the limit of detection (<0.55 μM) in all

Table 1 Plasma concentration of metabolites following 7 days of a methyl group-deficient diet moderately deficient in methionine (pre-rescue) and following supplementation with folate, betaine, or a combination of both (post-rescue)

	Folate ($n = 6$)		Betaine ($n = 6$)		Folate + betaine ($n = 6$)		P value[†]		
	Pre-rescue	Post-rescue	Pre-rescue	Post-rescue	Pre-rescue	Post-rescue	Rescue	Treatment	Interaction
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD			
Choline (μM)	5.53 ± 4.71	1.89 ± 2.14	9.51 ± 9.74	5.83 ± 9.34	5.54 ± 5.20	7.19 ± 6.34	ns	ns	ns
DMG (μM)	0.131 ± 0.262	0.071 ± 0.072	0.113 ± 0.084	8.43 ± 10.21	0.082 ± 0.108	5.87 ± 7.73	0.0167	ns	ns
Methionine (μM)	10.0 ± 3.0	12.0 ± 5.8	12.3 ± 3.7[a]	6.7 ± 1.6[b]	11.9 ± 3.9	9.4 ± 4.6	ns	ns	0.049
Homocysteine (μM)	32.1 ± 22.8	19.9 ± 12.4	26.6 ± 6.4	23.5 ± 11.9	31.4 ± 11.1	19.4 ± 10.1	0.0009	ns	ns
Cysteine (μM)	31.1 ± 9.5	34.9 ± 8.6	32.8 ± 5.3	32.6 ± 10.1	31.3 ± 6.6	37.3 ± 7.4	ns	ns	ns

ns not significant, *DMG* dimethylglycine

[†] Data were analyzed by two-way ANOVA with main effects of treatment (i.e., various methyl donors) and rescue (i.e., pre-/post-supplementation) and using Bonferroni posttests when main effects were identified. Labeled means in a row without a common letter differ

animals receiving folate supplementation alone while values for plasma betaine changed from below detection limit (<0.55 μM) to 146 ± 58 and 103 ± 64 μM in betaine, and folate + betaine-supplemented groups, respectively.

Phenylalanine infusion

There was a main effect of rescue on both the rate of phenylalanine oxidation ($P = 0.006$) (Table 2) and % phenylalanine oxidized ($P = 0.004$) (Fig. 2), with 20–46 % lower levels in both measures during the post-rescue infusion, with no treatment × rescue interaction. There were no differences in non-oxidative disposal, protein breakdown or phenylalanine flux following supplementation (Table 2).

Discussion

These data demonstrate that folate and betaine, alone or in combination, are able to lower phenylalanine oxidation, an indicator of whole-body protein synthesis, in piglets receiving a diet limited in methionine. Methionine metabolism is especially important during early development when higher levels of both protein synthesis and transmethylation via SAM create a high demand for methionine which must be partitioned between these two functions. In this study, we fed a methyl-deficient diet with a moderately deficient level of methionine for 6 days to minimize available methionine making it first limiting for protein synthesis. After 2 days of supplementation with folate, betaine, or both, we hypothesized that the increased remethylation will increase available methionine and stimulate whole-body protein synthesis, which was measured by indicator amino acid (i.e., phenylalanine) oxidation. Supplementation with folate, betaine, or a combination of both equally lowered the rate of phenylalanine oxidation and the percent of phenylalanine dose oxidized, but not non-oxidative phenylalanine disposal, suggesting that folate and betaine have an equal capacity to remethylate methionine for whole-body protein synthesis.

Although both folate and betaine function as remethylation nutrients, there are few studies comparing folate and betaine and their respective capacity to reform methionine. Experiments in rat hepatocytes have demonstrated that homocysteine is partitioned equally between both remethylation pathways [30]. Furthermore, following an intravenous bolus infusion of [$^2H_7$]methionine in folate- or choline-deficient rats, plasma [$^2H_4$]homocysteine was higher in folate-deficient animals during the 120-min experiment compared to both choline-deficient and control groups; however, levels of remethylated methionine in the plasma were not different among the three groups. In keeping with our findings, these results suggest an equal capacity for folate and betaine to remethylate methionine, while higher levels of [$^2H_4$]homocysteine following folate deficiency are likely the result of hepatic and renal localization of BHMT while MS is ubiquitous [31]. Our study adds to this body of knowledge by comparing the capacity of these two nutrients in neonatal animals demonstrating that both nutrients can equally reform methionine for whole-body protein synthesis when methionine is first limiting, as indicated by lower indicator amino acid oxidation.

In order to determine the capacity for folate and betaine to reform methionine, it was first necessary to limit the availability of these remethylation nutrients. To create a deficiency in these nutrients, we fed piglets an IG elemental diet moderately deficient in methionine and devoid of folate, betaine, and choline for 6 days. The effectiveness of the methyl group-deficient diet is evident by the lower plasma levels of relevant metabolites on day 7 compared to baseline. Although bacterial folate synthesis in the large intestine can contribute to plasma folate status [32], we still observed a significant 65 % reduction in plasma folate.

Table 2 Phenylalanine kinetics following a primed, constant infusion of L-[^{13}C]-phenylalanine in piglets after 6 days of receiving a methyl group-deficient diet moderately deficient in methionine (pre-rescue) and following supplementation with either folate, betaine, or a combination of both (post-rescue)

	Folate ($n = 6$)		Betaine ($n = 6$)		Folate + betaine ($n = 6$)		P value[†]		
	Pre-rescue	Post-rescue	Pre-rescue	Post-rescue	Pre-rescue	Post-rescue	Rescue	Treatment	Interaction
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD			
Phe_{ox} (μmol kg^{-1} h^{-1})	6.38 ± 2.82	4.35 ± 1.77	7.70 ± 3.90	4.56 ± 3.75	6.85 ± 2.12	4.79 ± 2.46	0.006	ns	ns
NOD (μmol kg^{-1} h^{-1})	477 ± 107	612 ± 156	498 ± 74	573 ± 111	544 ± 110	472 ± 74	ns	ns	ns
Flux (μmol kg^{-1} h^{-1})	483 ± 108	616 ± 157	505 ± 75	577 ± 111	550 ± 111	477 ± 74	ns	ns	ns
PB (μmol kg^{-1} h^{-1})	275 ± 108	408 ± 157	297 ± 75	370 ± 111	343 ± 111	268 ± 74	ns	ns	ns
Intake (μmol kg^{-1} h^{-1})	208 ± 0	208 ± 0	208 ± 0	208 ± 0	208 ± 0	208 ± 0	ns	ns	ns

NOD non-oxidative disposal, *ns* not significant, *PB* protein breakdown, Phe_{ox} rate of phenylalanine oxidation

[†] Data were analyzed by two-way ANOVA with main effects of treatment (i.e., various methyl donors) and rescue (i.e., pre-/post-supplementation); only rescue was significant for all outcomes so these *P* values are presented

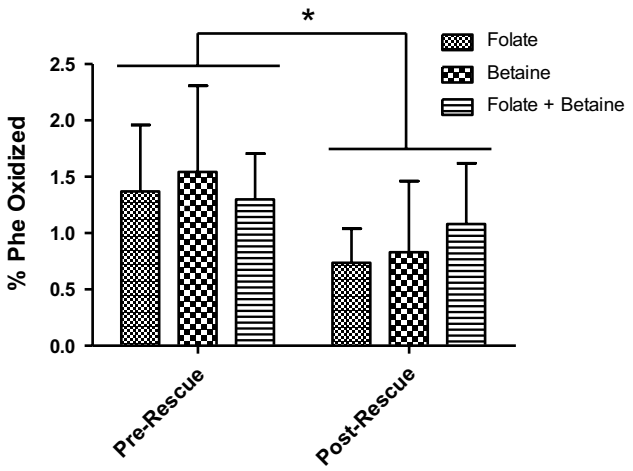

Fig. 2 Effect of dietary methyl donor supplementation on percentage of phenylalanine dose oxidized during L-[1-^{13}C]-phenylalanine infusion. Means and standard deviation presented and analyzed using two-way ANOVA. *Asterisk* represents main effect of rescue ($P < 0.05$). $n = 6$ per group. *Phe* phenylalanine

Moreover, in addition to a 70 % lower plasma concentration of choline, betaine was depleted by day 7.

Studies have demonstrated that folate deficiency leads to an increase in plasma homocysteine concentrations [33, 34]; however, we did not observe this outcome, possibly as a result of the concurrent ~30 % restriction in dietary methionine, potentially limiting flux through transmethylation and subsequent formation of homocysteine. Deletion of the BHMT gene in mice leads to higher levels of homocysteine in the plasma, and interestingly, supplementation with folate does not lead to normalization of homocysteine concentrations in these animals [35], highlighting the importance of betaine as a remethylation nutrient. We observed a "rescue" effect of methyl nutrients to lower homocysteine concentrations, but could not discern the effectiveness of folate versus betaine on this outcome. Interestingly, there was no main effect of rescue or methyl donor on methionine concentration; however, there was an interaction effect with lower methionine in the betaine-supplemented group post-rescue. Because BHMT is primarily found in the liver and MS is ubiquitous, it is possible that methionine reformed via hepatic BHMT was primarily used in the liver and consequently less was transported in the plasma. Although we did not demonstrate higher plasma methionine concentrations post-rescue, we have determined higher rates of remethylation post-rescue for all groups using [^{13}C,^{2}H-methyl]methionine [36]; it is likely newly remethylated methionine is immediately used for protein synthesis and/or transmethylation given the methionine-deficient state of the piglets.

Our data demonstrate that folate and betaine, either individually or together, equally lowered indicator amino acid oxidation as well as lowered plasma homocysteine in piglets receiving a diet that was first limited in methionine, suggesting that these nutrients have an equal capacity to remethylate methionine. However, in spite of changes in oxidation, there were no statistical differences in phenylalanine NOD, consistent with previous methionine requirement studies using phenylalanine oxidation in piglets [4, 19]. Although NOD is supposed to reflect protein synthesis, it is typically highly variable in such studies. Because NOD is a stochastic calculation from highly variable flux measurements minus very low oxidation measurements (<2 % of flux), NOD is more reflective of the flux variability rather than treatment effect and is not a very responsive outcome compared to oxidation [4, 19].

The observation that folate and betaine can affect the availability of methionine is important as the levels of both sulfur amino acids and remethylation nutrients are variable in the neonatal diet. Furthermore, because ~2.5 % of newborns have hyperhomocysteinemia (>15 μmol l^{-1}) [37], the equal capacity for both remethylation nutrients to lower plasma homocysteine is an important outcome when considering interventions to lower homocysteine in neonates. These data make it important for future studies to further investigate the methionine requirement of the neonate in relation to other nutrients that converge on methionine metabolism.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Authorship L.E.M., J.L.R., R.F.B., and J.A.B. were involved in formulating the research question and designing the experiment. L.E.M. and J.L.R. carried out the experiments, and L.E.M., J.L.R., S.V.H., and E.W.R. conducted the analytical work. L.E.M., J.L.R., R.F.B., and J.A.B. analyzed and interpreted the data. L.E.M. and R.F.B. drafted the manuscript, and all authors read and reviewed the final version.

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