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Substitution of Dietary Sulfur Amino Acids by DL-2-Hydroxy-4-Methylthiobutyric Acid Reduces Fractional Glutathione Synthesis in Weaned Piglets

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ABSTRACT

Background: Cys is limiting for reduced glutathione (GSH) synthesis and can be synthesized from Met. We hypothesized that the dietary Met hydroxyl analogue DL-2-hydroxy-4-methylthiobutyric acid (DL-HMTBA) affects Cys and GSH metabolism and oxidative stress defense differently than Met.

Objective: The objective was to elucidate whether DL-HMTBA supplementation of a Met-deficient diet affects Cys flux, GSH fractional synthetic rate (FSR), and the basal oxidative stress level relative to Met supplementation in pigs.

Methods: Twenty-nine male German Landrace piglets aged 28 d were allocated to 3 dietary groups: a basal diet limiting in Met (69% of Met plus Cys requirement) supplemented with either 0.15% L-Met (LMET; $n = 9$), 0.15% DL-Met (DLMET; $n = 11$), or 0.17% DL-HMTBA (DLHMTBA; $n = 9$) on an equimolar basis. At age 54 d the pigs received a continuous infusion of [1-^{13}C]-Cys to calculate Cys flux and Cys oxidation. After 3 d, GSH FSR was determined by [2,2-$^{2}H_2$]-glycine infusion, and RBC GSH and oxidized GSH concentrations were measured. At age 62 d the animals were killed to determine hepatic mRNA abundances of enzymes involved in GSH metabolism, GSH concentrations, and plasma oxidative stress defense markers.

Results: The Cys oxidation was 21–39% and Cys flux 5–15% higher in the fed relative to the feed-deprived state ($P < 0.001$). On average, GSH FSR was 49% lower ($P < 0.01$), and RBC GSH and total GSH concentrations were 12% and 9% lower, respectively, in DLHMTBA and DLMET relative to LMET pigs ($P < 0.05$). In the feed-deprived state, Gly flux, the GSH:oxidized glutathione (GSSG) ratio, RBC GSSG concentrations, plasma oxidative stress markers, and the hepatic GSH content did not differ between groups.

Conclusions: Although GSH FSR was higher in LMET compared with DLMET or DLHMTBA feed-deprived pigs, these differences were not reflected by lower oxidative stress markers and antioxidant defense enzymes in LMET pigs. *J Nutr* 2020;150:722–729.

Keywords: DL-2-hydroxy-4-methylthiobutyric acid, cysteine, glycine, fluxes, glutathione synthesis, pigs

Introduction

Reduced glutathione (GSH) is an intracellular antioxidant whose thiol moiety can act as scavenger for oxidizing molecules thereby protecting cells from oxidative stress and toxic xenobiotics. GSH is essential for early embryogenesis (1), whereas GSH deficiency can compromise integrity and function of specific brain regions in mice (2). Children with severe protein-energy malnutrition as well as preterm and low-birth-weight infants and piglets have reduced erythrocyte GSH concentrations and elevated levels of oxidative stress (3–5). The synthesis of GSH (γ-L-glutamyl-L-cysteinylglycine) is catalyzed by the enzymes glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS), and the availability of Cys as substrate for GCL is considered the limiting factor for GSH synthesis, at least in rodents (6). Although several tissues are capable of Cys synthesis, dietary supplementation with Cys (3) has been shown to restore a normal GSH concentration and synthesis rate in children with edematous malnutrition (7).

The intracellular precursor for Cys synthesis is homocysteine (Hcy). Hcy in turn is synthesized from Met via the transmethylation pathway and is remethylated to form L-Met in the Met cycle. Thus, Met as an indispensable amino acid (AA) serves as

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an important precursor for Cys and GSH synthesis. Accordingly, dietary restriction of Met and Cys reduces GSH synthesis rate in adult humans (8), whereas dietary L-Met supplementation to low-birth-weight piglets increased the amount of GSH in the muscle (9) and the jejunum (10). However, oral L-Met supplementation to children with severe acute malnutrition had no effect on GSH synthesis rates but increased the plasma Cys flux (11). The GSH synthesis rate is increased when growing pigs are immune-challenged (12). Although the liver is the major site for Met transmethylation and the conversion of Hcy to Cys in the transsulfuration pathway (13), the gastrointestinal tract of infant pigs can metabolize 20–30% of dietary L-Met intake and accounts for ~25% of whole body transmethylation and transsulfuration (13–15). These results indicate the importance of the first-pass Met metabolism not only for the intestinal tract but also for its contribution to whole body transsulfuration.

It was speculated that dietary supplementation with the cheaper Met analogue DL-2-hydroxy-4-methylthiobutyric acid (DL-HMTBA) compared with DL-Met favors transsulfuration over remethylation both in the intestine and liver of piglets (14). This was deduced from elevated arterial taurine (Tau) concentrations in DL-HMTBA–supplemented piglets (16). A greater transsulfuration has been assumed to account for a higher jejunal GSH content and lower oxidized glutathione (GSSG):GSH ratio in piglets supplemented with DL-HMTBA relative to DL-Met (16). However, our recent studies using stable isotope tracer approaches clearly demonstrated that piglets supplemented with DL-HMTBA had lower whole-body transsulfuration rates relative to counterparts supplemented with L- and DL-Met (17). Despite clear evidence that this dietary Met analogue affects transsulfuration and remethylation to different extents, its impact on GSH synthesis has not been established yet. The liver is the primary producer of GSH released into plasma (6), but the majority of GSH in the circulation originates from de novo synthesis in RBCs. Our hypothesis was that piglets fed diets supplemented with DL-HMTBA would show a lower GSH synthesis rate in RBCs as well as diminished hepatic GSH synthesis capacity, both causing compensatory upregulation of oxidative stress defense enzymes. The objective of the present study was to quantify the impact of dietary DL-HMTBA relative to L- and DL-Met supplementation on Cys flux (Q_{Cys}), Cys oxidation (Ox_{Cys}), and GSH fractional synthetic rate (FSR) in RBCs, and the transcription of enzymes regulating GSH metabolism in the liver and antioxidant defense enzymes in plasma of weaned pigs.

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Abbreviations used: AA, amino acid; ACN, acetonitrile; AP, atom percentage; DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; dROM, reactive oxygen metabolite; FSR, fractional synthetic rate; GCL, glutamate-cysteine ligase; GGT, γ-glutamyltransferase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSS, glutathione synthetase; GSSG, oxidized glutathione; Hcy, homocysteine; IE, isotopic enrichment; IR, infusion rate; MBB, monobromobimane buffer; Ox_{Cys}, cysteine oxidation; PSEM, pooled SEM; Q_{Cys}, cysteine flux; Q_{Gly}, plasma glycine flux; SOD, superoxide dismutase; Tau, taurine; TBARS, thiobarbituric acid substance; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$.

Methods

Animals and diets

The study protocol was approved by the Animal Care Committee of the Ministry of Nutrition, Agriculture, Forestry and Fishery, Rostock, Germany (LALLF 7221.3–1-037/14). Thirty-three weaned piglets (age 28 d; male; German Landrace; mean body weight 9.35 ± 0.22 kg) from 14 sows of the institutional experimental pig facility were randomly allocated to 3 different isoenergetic and isonitrogenous diets. An L-Met–deficient basal diet, formulated based on corn, soybean meal, peas, and whey powder to meet 69% of Met + Cys recommendations (0.47% standardized ileal digestibility of Met + Cys, as-fed basis) for 11–25-kg pigs, was supplemented on an equimolar basis with either 0.15% L-Met (LMET; $n = 11$), 0.15% DL-Met (DLMET; $n = 11$), or 0.17% DL-HMTBA (DLHMTBA; $n = 11$) to provide 90% of Met + Cys requirement (18) (**Supplemental Tables 1** and **2**). The dietary Cys content was analyzed to be 0.29% and did not differ among diets. All diets were adequately supplemented with minerals and vitamins. Animals were fed 95% of the recommended dietary allowance in 2 equal meals (at 06:00 and 18:00) to ensure comparable feed intake in all groups. Piglets were individually housed in metabolic cages (0.5 m^2) with ad libitum access to water at 24°C with a 12:12-h dark/light cycle. Individual feed intake was recorded daily. At the age of 41 d, after being fed the experimental diets for 9 d, piglets were feed-deprived overnight, anesthetized (20 mg/kg ketamine, 2 mg/kg xylazine, and 1–2 mg/kg diazepam), and fitted with catheters implanted in the arteria carotis communis and vena jugularis externa dextra (17). From days 42 to 44, piglets received a daily intravenous application of 50 mg/kg metamizole-Na and 24 mg/kg sulfadimidine-Na. Normal feed intake of piglets was resumed within 4 d postsurgery. Due to catheter clogging, Cys and Gly tracer analyses, as well as GSH analysis in RBCs, could only be performed for 28, 24, and 26 piglets, respectively.

Tracer application, blood and tissue sampling

Under our study conditions, the average body weights were 17.7, 16.8, and 17.2 kg [pooled standard error of the mean (PSEM) = 0.7 kg] at the age of 52 d ($P = 0.525$), and 23.6, 22.4, and 22.8 kg (PSEM = 0.7 kg) at the age of 60 d ($P = 0.547$) for the LMET, DLMET, and DLHMTBA groups, respectively.

In general, basal arterial blood samples were collected after a 14-h overnight feed withdrawal at age 54 and 57 d for analysis of background isotopic enrichment in lithium-heparin–containing tubes, as well as at age 57 d and 62 d for GSH and oxidative stress marker concentrations in K-EDTA–containing tubes 20 and 10 min before start of tracer infusion to obtain plasma.

Cysteine kinetics.

Starting at 08:00 at age 54 d, pigs received a primed, continuous intravenous 9 h-infusion of [1-^{13}C]-Cys [99 atom percentage (AP) carbon-13; Euriso-Top]. The priming dose was 7.5 μmol/kg and the infusion rate 7.5 μmol/(kg·h) through a 0.2-μm sterile filter. From 0 to 3 h of the tracer infusion, feed was withheld and arterial blood samples (2.5 mL) were collected every 30 min (feed-deprived state). Half-hourly meals (7% of daily allowance each) were fed until the end of infusion, and arterial blood sampling was continued from 6 to 9 h of infusion every 30 min (fed state).

Glycine kinetics.

At the age of 57 d, pigs received an intravenous priming dose (25 μmol/kg) of labeled Gly ([2,2-$^2H_2$]-Gly; 98.7 AP hydrogen-2; Sigma-Aldrich), followed by a constant infusion [25 μmol/(kg·h)] for 8 h. Arterial blood samples were drawn 4, 5, 6, 7, and 8 h after the start of infusion. At 0, 5, and 8 h after the start of the infusion further arterial blood samples were collected in K-EDTA–containing tubes to obtain RBCs after centrifugation (10 min; 3500 × *g*; 4°C). The RBCs were washed 3 times with 0.9% saline and stored at −80°C.

After overnight feed deprivation, an arterial blood sample was taken in the morning at 62 d of age and the pigs were killed by electrostunning

and subsequent exsanguination. Liver tissue (~5 g) was collected and immediately snap-frozen in liquid nitrogen.

Plasma ^{13}C-Cys enrichment and Cys flux

Plasma Cys was converted to its *tert*-butyldimethylsilyl derivative using a similar protocol to Rasch et al. (17). Diagnostic ions of the Cys derivative were detected at an *m/z* of 406 (M + 0) and 407 (M + 1). The mean enrichments for [1-^{13}C]-Cys in the feed-deprived state were determined from 2 to 3 h, and in fed-state enrichments from 6 to 9 h relative to the start of the tracer infusion. Whole body Ox_{Cys} and Q_{Cys} [both expressed in μmol/(kg·h)] were calculated as described recently (19, 20, 21) using the following equations:

$$Ox_{Cys} = V_{13CO2} \times \left(1/IE_{plasma} - 1/IE_{13C-Cys-infusate}\right) \quad (1)$$

$$Q_{Cys} = IR \times \left[\left(IE_{13C-Cys-infusate}/IE_{plasma}\right) - 1\right] \quad (2)$$

where V_{13CO2} is the produced volume of ^{13}C-carbon dioxide released from blood, IR is the [1-^{13}C]-Cys tracer infusion rate [μmol/(kg·h)], $IE_{13C\text{-}Cys\text{-}infusate}$ is the infusate isotopic enrichment [mole percent excess (MPE)], and IE_{plasma} is the isotopic enrichment of [1-^{13}C]-Cys [M + 1] in the plasma. Blood was treated with 10% lactic acid, released ^{13}C-carbon dioxide measured using an isotope ratio mass spectrometer (DELTAplus XL; Thermo Fisher Scientific), and V_{13CO2} calculated as described (17, 22).

$^2H_2$-Gly enrichment in erythrocyte GSH, plasma free Gly, and erythrocyte free Gly

Incorporation of labeled Gly into the GSH of intact RBCs was determined according to Sekhar et al. (23). Briefly, 150 μl RBCs were mixed with 250 μL monobromobimane buffer (MBB) for 45 min at 4°C followed by addition of perchloric acid (800 μL, 1.5 M). After centrifugation (10 min; 3500 × *g*; 4°C) the supernatant was neutralized with 400 μL 2 M K_2CO_3 at 4°C. The MBB-GSH derivative was purified by semipreparative HPLC (Series 1200/1260; Agilent) on a HyperClone octadecylsilane (C18) 120-Å column (250 × 4 mm; Phenomenex) at 30°C with a flow of 1.1 mL/min and gradient elution [eluent A: acetonitrile (ACN); eluent B: 1% acetic acid; 0 min, 5% A; 20 min, 20% A; 25 min, 100% A; 30 min, 100% A; 32 min, 5% A; 35 min, 5% A]. The detection of GSH was performed by absorption at 394 nm and fluorescence at 490 nm. The combined GSH-containing fractions of 10 injections were collected and dried at 60°C under nitrogen, hydrolyzed with 1 mL 6 N HCl at 110°C for 4 h, and dried again. GSH-bound Gly as well as plasma and free RBC Gly was derivatized with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide and separated by GC-MS as described above. Positive chemical ionization was applied, and resulting diagnostic ions of $^2H_2$-Gly enrichment were *m/z* 304 (M + 0), 305 (M + 1), and 306 (M + 2). The mean tracer enrichment was determined from hourly samples between 4 and 8 h from the start of infusion. In RBCs the free $^2H_2$-Gly steady-state enrichment was reached 5 h after starting the tracer infusion.

Calculation of GSH FSR and Gly flux

The FSR of GSH, that is, the percentage of GSH in RBCs that is synthesized in a day (%/d), was calculated according to Sekhar et al. (23):

$$FSR_{GSH}\left(\%/d\right) = \left(IE_{t8} - IE_{t5}\right) / \left(IE_{RBC} \times \left(t_8 - t_5\right)\right) \times 24\,h/d \times 100 \quad (3)$$

where IE_{t5} and IE_{t8} is the isotopic enrichment of GSH-bound Gly isolated from RBCs at time (t) 5 and 8 h after the start of the tracer infusion, and IE_{RBC} is the isotopic enrichment of RBC free $^2H_2$-Gly under steady-state conditions.

Plasma Gly flux (Q_{Gly}) expressed in mmol/(kg·h) was calculated as:

$$Q_{Gly} = IR \times \left[\left(IE_{2H2-Gly-infusate}/IE_{plasma}\right) - 1\right] \quad (4)$$

where IR is the [$^2H_2$]-Gly tracer infusion rate [mmol/(kg·h)], $IE_{2H2\text{-}Gly\text{-}infusate}$ is the infusate enrichment, and IE_{plasma} is the isotopic enrichment of [$^2H_2$]-Gly in the plasma.

Erythrocyte and liver GSH and GSSG and plasma AA concentrations

Concentrations of GSH and GSSG in RBCs and liver were analyzed as fluorescent *ortho*-phthaldialdehyde derivatives according to Kuhla et al. (24) with slight modifications. Briefly, 30 μL thawed RBCs were mixed on ice with 60 μL of iodoacetic acid (25 mg/mL in 0.5 M bicine buffer pH 9.0), diluted with water to 600 μL, and analyzed by HPLC (Series 1200; Agilent), allowing the coanalysis of GSSG and GSH in 1 run. A 250 × 4-mm HyperClone C18 120-Å column (Phenomenex) and a binary gradient (eluent A: ACN/methanol/water = 45:45:10; eluent B: 40 mM phosphate buffer pH 7.8; 0 min, 1% A; 8 min, 9% A; 15 min, 14% A; 20 min, 100% A) was used. Total GSH concentration was calculated as the sum of GSH and GSSG concentrations. Sample preparation and analysis of plasma AAs were performed as described earlier (24).

Real-time qRT-PCR

Transcriptional hepatic mRNA abundances of genes involved in GSH metabolism were analyzed by quantitative real-time PCR according to the MIQE guidelines (25). Briefly, RNA was isolated by using TriFast reagent (Peqlab) and RNeasy Mini kit (Qiagen). RNA quality was assessed using an Agilent 2100 Bioanalyzer, yielding RNA integrity number factors between 7.6 and 9.8 (mean 9.1 ± 0.1). First-strand cDNA synthesis was performed using 750 ng total RNA, 2400 U RevertAid Reverse Transcriptase (Thermo Fisher Scientific), and 250 pmol random primers. The cDNA was purified with High Pure PCR Product Purification Kit (Roche). Primer sequences are shown in **Supplemental Table 3**. One reaction contained 2 μL diluted cDNA, 3 μL H_2O, 0.5 μL of each 4-μM primer, and 5 μL 2 × buffer SensiFAST SYBR No-Rox Mix (Bioline) and was carried out in triplicates using a LightCycler 96 (Roche). Primer products were sequenced and correct sequence was confirmed. The efficiency of amplification was calculated using LinRegPCR Software (version 2014.4; Academic Medical Center), yielding efficiency values >1.8 (Supplemental Table 3). Data were quantified by qbasePLUS software (Biogazelle) and normalized to the most stable genes, which were ribosomal protein L4 and S18 (*RPL4*, *RPS18*) and peptidylprolyl isomerase A (*PPIA*) (M-value 0.349; V-value 0.141) according to Uddin et al. (26) and Zhang et al. (27).

Oxidative stress and antioxidant defense markers in plasma

At age 57 and 62 d, plasma concentrations of thiobarbituric acid substances (TBARSs) and reactive oxygen metabolites (dROMs) were analyzed using kits (TBARS: #327131; R&D systems; dROMs: #V1504; Diacron Labs). To measure plasma 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) concentration, 1 mL plasma was treated with butylated hydroxytoluene and frozen at −80°C. After thawing, 8-iso-$PGF_{2\alpha}$ was analyzed using a kit (#16,290; Cayman) as described (28). The concentrations of vitamins A (retinol) and E (tocopherol) in serum were determined by a commercial laboratory (Synlab.vet) using routine HPLC methods. The activity of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were analyzed in full blood obtained after overnight feed deprivation at ages 57 and 62 d using a RANSOD (#358385) and RANSEL (#350995) kit (Randox Laboratories), respectively.

Statistical analysis

All data analyses were performed with SAS software for Windows, version 9.4 (SAS Institute Inc.). The data were analyzed by repeated measures ANOVA with the MIXED procedure of SAS/STAT software.

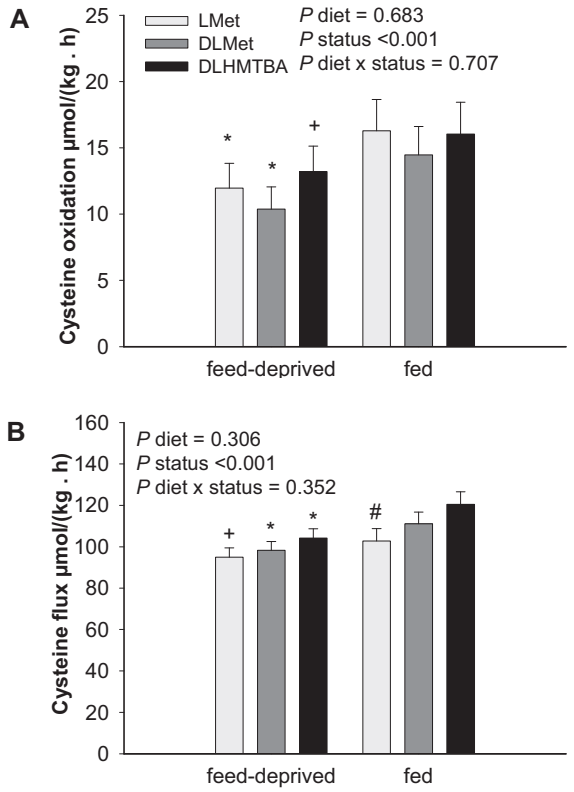

FIGURE 1 Whole body cysteine oxidation (A) and cysteine flux (B) in 54-d-old pigs after 14-h feed deprivation having been fed a diet supplemented with either L-Met (+0.15%; LMET, $n = 9$); DL-Met (+0.15%; DLMET, $n = 10$), or DL-2-hydroxy-4-methylthiobutyric acid (+0.17%; DLHMTBA, $n = 9$) on an equimolar basis to a Met-deficient basal diet. Values are least-square means ± SE. *Different from fed, $P < 0.05$. $^{+}$Different from fed, $P < 0.1$, ANOVA F test. $^{\#}$Trend relative to DLHMTB in fed status, $P < 0.1$, Tukey–Kramer test.

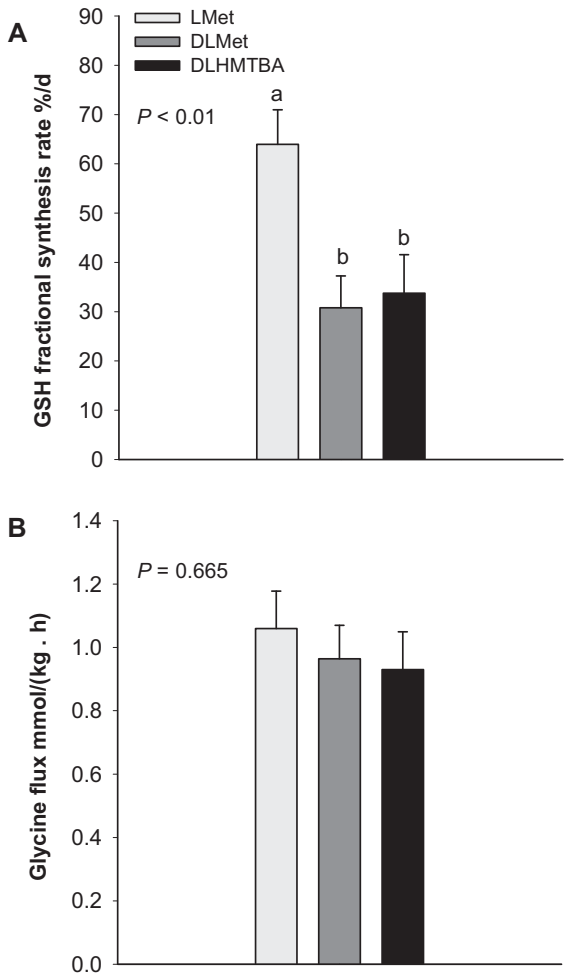

FIGURE 2 Fractional synthetic rate of reduced glutathione (GSH) (A) and Gly flux (B) in 57-d-old overnight feed-deprived pigs fed diets containing differing Met sources starting at weaning (age 28 d). The Met-deficient basal diet (69% of SID Met + Cys recommendation) was supplemented with either L-Met (+0.15%; LMET, $n = 7$); DL-Met (+0.15%; DLMET, $n = 10$), or DL-2-hydroxy-4-methylthiobutyric acid (+0.17%; DLHMTBA, $n = 7$). Values are least-square means ± SE. Labeled bars without a common letter differ, $P < 0.05$, Tukey–Kramer test. SID, standardized ileal digestibility.

The ANOVA model for Cys flux and oxidation contained the fixed effects of diet (LMET, DLMET, DLHMTBA), status (fed, feed-deprived), and the interaction diet × status. The model for analysis of Gly flux, FSR of GSH, hepatic transcript abundances, and vitamin and AA concentrations contained the fixed effect of diet (LMET, DLMET, DLHMTBA). The model for the analysis of oxidative stress markers and 8-iso-$PGF_{2\alpha}$ contained the fixed effects of diet (LMET, DLMET, DLHMTBA), age (57 and 62 d), and the interaction diet × age. In all models the sow was considered as random effect to model the dependency between siblings. Repeated measurements on the same animal were taken into account by the repeated variable status using an unstructured type for the block diagonal residual covariance matrix. Differences among dietary groups and fed compared with feed-deprived state within a group but also between days of age were analyzed using the Tukey–Kramer test. Differences were considered significant if $P \leq 0.05$ and a trend if $P \leq 0.1$.

Results

Cys flux

To assess the oxidation and flux rate of Cys as critical elements determining its availability as rate-limiting AA for GSH synthesis, we performed a [1-^{13}C]-Cys tracer infusion study in pigs fed diets with 3 different Met sources from weaning at age 28 d onwards. Under conditions of 14-h overnight fasting, Ox_{Cys} and Q_{cys} were similar among pigs supplemented with L-Met, DL-Met, or L-HMTBA (Figure 1A, B). During the fed state, the L-Met– and DL-Met–supplemented diets increased Ox_{Cys} by 35% and 39% relative to the fasting state ($P < 0.05$), whereas DL-HMTBA supplementation tended to increase Ox_{Cys} by only 21% ($P < 0.1$). The ^{13}C-Cys tracer study further revealed that DL-Met and DL-HMTBA supplementations increased Q_{Cys} by 13–15% ($P < 0.05$), whereas the L-Met added diet tended to increase Q_{Cys} by 5% ($P < 0.10$) relative to the feed-deprived state. The increase was greatest with the ingestion of the DL-HMTBA–supplemented diet, and Q_{Cys} tended to be 17% higher in DL-HMTBA compared with L-Met pigs ($P = 0.1$), suggesting a trend for greater Cys flux with DL-HMTBA supplementation.

FSR of GSH and Gly flux in RBCs

To test whether dietary DL-HMTBA supplementation affects GSH synthesis, pigs received a $^{2}H_2$-Gly tracer infusion to quantify the FSR of GSH in RBCs. After feed deprivation, pigs on a diet supplemented with either DL-HMTBA or DL-Met had on average a 49% lower FSR of GSH compared with animals supplemented with L-Met ($P < 0.01$) (Figure 2A). However,

Q_{Gly} was not different among dietary groups (Figure 2B). We next examined whether the lower GSH FSR in DLHMTBA pigs would affect RBC GSH concentrations after feed deprivation. Relative to L-Met–supplemented pigs, the GSH concentration in RBCs was 12% lower in DLMET ($P < 0.05$) and tended to be 11% lower in DLHMTBA ($P = 0.07$) pigs (**Figure 3**A). The total GSH concentrations in RBCs was 8% lower in the DLMET and 11% lower in the DLHMTBA groups relative to the LMET group ($P < 0.05$) (Figure 3B). However, RBC GSSG concentrations and the GSH:GSSG ratio did not differ among groups (Figure 3C, D).

Hepatic transcripts controlling GSH homeostasis and liver GSH content

We next analyzed the abundance of hepatic transcripts involved in liver, bile, and plasma GSH homeostasis. The hepatic *GSS*, glutathione reductase (*GR*), and gamma-glutamyltransferase (*GGT*) mRNA abundances were not different among groups, although the mRNA expression of the glutamate-cysteine ligase (*GCL*) catalytic subunit, ligating intracellular Cys and Glu, was more abundant in DLHMTB than in LMET pigs ($P < 0.05$) (**Table 1**). Furthermore, mRNA expression of *GPX*, oxidizing GSH to GSSG, was 1.33-fold greater in DLHMTBA than in DLMET pigs ($P < 0.05$), with intermediate values in LMET pigs. However, the GSH content in liver was not different among groups [3.20 ± 0.33, 2.69 ± 0.28, and 2.51 ± 0.33 μg/g liver wet weight for LMET ($n = 9$), DLMET ($n = 11$), and DLHMTBA ($n = 9$) pigs, respectively].

Plasma AA, oxidative stress markers, and antioxidant enzymes

Plasma Cys, Met, Gln, Gly, and Tau concentrations were unaffected by the dietary Met source (**Table 2**). Plasma oxidative stress markers such as TBARS, dROMs, and 8-iso-$PGF_{2\alpha}$ were not different among dietary treatments, but dROM concentrations increased from day 57 to 62 in all groups by a factor of 1.3 ($P < 0.05$) (Table 2). The concentrations of vitamins A and E and the plasma GPX activity were not different among groups. However, plasma SOD activity decreased from age 57 d to 62 d in LMET and DLMET pigs by 29% and 17%, respectively ($P < 0.05$), and tended to be 1.22-fold greater in DLHMTBA than LMET animals ($P < 0.1$).

Discussion

The hypothesis was that piglets fed Met-limited diets supplemented with DL-HMTBA would show a lower GSH synthesis rate in RBCs, downregulation of hepatic GSH production, and compensatory upregulation of oxidative stress defense enzymes relative to pigs with L-Met or DL-Met supplementation. The primary outcome of our study was that DLHMTBA and DLMET compared with LMET pigs had a lower GSH FSR in RBCs after overnight feed deprivation reflecting the postabsorptive state. In parallel, GSH and total GSH concentrations in RBCs were lower or tended to be lower in DLHMTBA and DLMET pigs relative to LMET pigs, whereas the GSSG concentration and GSH:GSSH ratio as an indicator of the redox potential remained unaffected. There was a tendency for a higher plasma SOD concentration in pigs aged 62 d fed the DLHMTBA diet, which might exacerbate the impact of low GSH concentrations on endogenous H_2O_2 regulation in DLHMTBA pigs at this age. However, the oxidative stress metabolite concentrations of TBARS, dROMs, and 8-iso-$PGF_{2\alpha}$ were maintained at the level

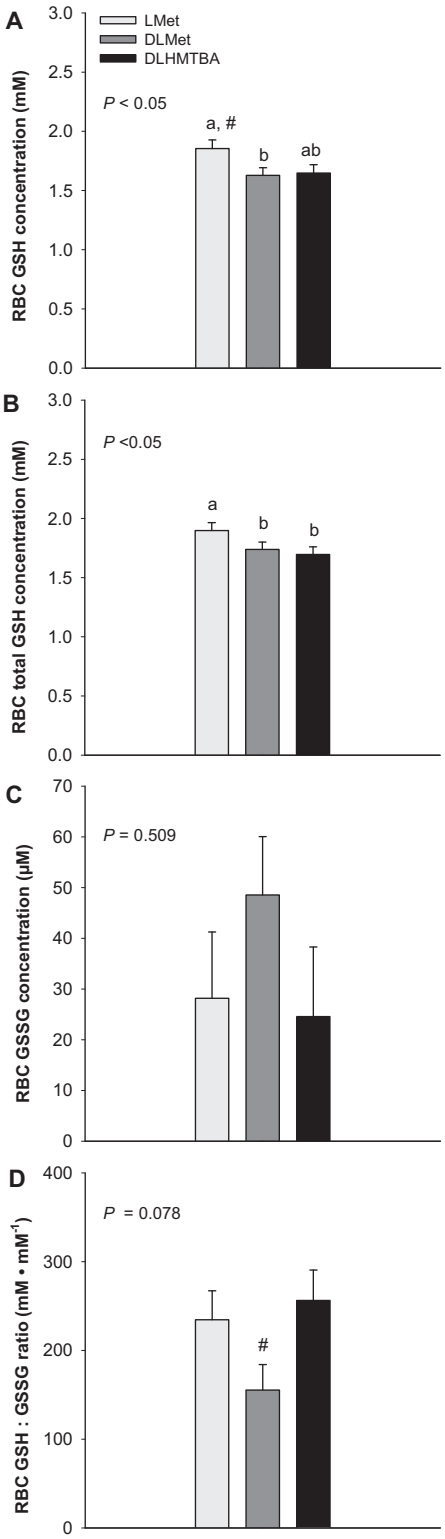

FIGURE 3 Concentrations of GSH (A), total GSH (B), GSSG (C), and the GSH:GSSG ratio (D) in RBCs of 57-d-old pigs after overnight feed deprivation, having been fed diets containing differing Met sources starting at weaning (age 28 d). The Met-deficient basal diet (69% of SID Met + Cys recommendation) was supplemented with either L-Met (+0.15%; LMET, $n = 8$), DL-Met (+0.15%; DLMET, $n = 10$), or DL-2-hydroxy-4-methylthiobutyric acid (+0.17%; DLHMTBA, $n = 8$). Values are least-square means ± SE. Labeled bars without a common letter differ, $P < 0.05$, Tukey–Kramer test. #Trend relative to DLHMTB, $P < 0.1$, Tukey–Kramer test. GSH, reduced glutathione; GSSG, oxidized glutathione; SID, standardized ileal digestibility.

TABLE 1 Relative mRNA abundances of genes linked to GSH synthesis and oxidative stress status in the liver of 62-d-old pigs (after 14-h overnight feed deprivation) fed diets containing differing Met sources starting at weaning (age 28 d)[1]

Gene	LMET	DLMET	DLHMTBA	*P* value[2]
GSS	1.01 ± 0.05	0.96 ± 0.05	1.08 ± 0.06	<0.05
GPX	1.38 ± 0.09[a, b]	1.24 ± 0.09[b]	1.57 ± 0.10[a]	<0.001
GR	1.57 ± 0.08	1.50 ± 0.08	1.63 ± 0.10	0.394
GCL	0.86 ± 0.06[b]	0.98 ± 0.06[a,b]	1.11 ± 0.05[a]	<0.001
GGT	1.10 ± 0.11	1.03 ± 0.11	1.13 ± 0.12	0.354
OPLAH	2.13 ± 0.12[a]	1.68 ± 0.13[b]	1.81 ± 0.14[a, b]	<0.001
SOD1	1.28 ± 0.08	1.21 ± 0.07	1.32 ± 0.08	0.359
SOD2	1.63 ± 0.17	1.31 ± 0.16	1.65 ± 0.17	<0.05

[1] All values are least-square means ± SE in arbitrary units and normalized to the most stable reference genes ribosomal protein L4 and S18 (*RPL4, RPS18*) and peptidylprolyl isomerase A (*PPIA*). Met-deficient basal diet (69% of SID Met + Cys recommendation) supplemented with either L-Met (+0.15%; LMET, $n = 9$); DL-Met (+0.15%; DLMET, $n = 11$), or DL-2-hydroxy-4-methylthiobutyric acid (+0.17%; DLHMTBA, $n = 8$). *GCL*, glutamate-cysteine ligase; *GGT*, gamma-glutamyl transferase; *GPX*, glutathione peroxidase; *GR*, glutathione reductase; GSH, reduced glutathione; *GSS*, glutathione synthetase; *OPLAH*, oxoprolinase; SID, standardized ileal digestibility;*SOD1*, copper-zinc superoxide dismutase; *SOD2*, manganese superoxide dismutase.
[2] ANOVA *F* test.

of L-Met and DL-Met supplementation. Increased SOD activity but unchanged SOD transcript amounts were also observed in the liver of Met-deficient pigs (29), further indicating a deficient Met status with DL-HMTBA supplementation. These results suggest that feeding young pigs a diet supplemented with DL-HMTBA or DL-Met compared with a diet supplemented with L-Met can reduce the synthesis of GSH after feed deprivation, but neither attenuates the systemic antioxidant capacity nor affects the oxidative stress level. Indeed, when piglets consumed ad libitum, Zeitz et al. (30) demonstrated that the effects of dietary L-Met and DL-Met supplementation were not different in regard to performance, gut morphology, and GSH concentration in liver and small intestine. In addition, plasma AA concentrations, mRNA expressions of 8 myogenesis-related genes in muscle as well as growth rates of pigs fed diets supplemented with DL-Met or L-Met were not different, indicating 100% equivalence in growth and protein synthesis of DL-Met and L-Met pigs (30–33).

Under feed-deprived conditions, the rates of Cys flux and Cys oxidation did not differ among dietary groups. When L-Met– and DL-Met–supplemented diets were ingested, Cys oxidation increased to 135% and 139% of the feed-deprived values; however, Cys oxidation tended to increase to only 121% with the DL-HMTBA diet relative to the feed-deprived state. Greater Cys oxidization could be because more Cys was available from the Met metabolism of LMET and DLMET than DLHMTBA pigs. Indeed, Cys oxidation was greater when young adults ingested a diet containing both Met and Cys compared with a diet containing Met alone (19). Another reason for the lesser stimulation of Cys oxidation in DLHMTBA pigs can be explained by our earlier observation that a DL-HMTBA–supplemented diet increases remethylation of Hcy, and thus Hcy is less available for transsulfuration (17).

Compared with the feed-deprived state, Cys flux during the fed state was higher for DLMET and DLHMTBA and tended to be higher in LMET pigs; however, Cys flux was not significantly affected by the Met source. In the present study we measured Cys flux when pigs were 54 d old and observed similar Cys fluxes as in 47-d-old pigs (17). However, at the younger age, the Cys flux tended to be higher in the fed state only in DLMET pigs but not in DLHMTBA pigs (17). The tendency for a greater Cys flux in DL-HMTBA–fed compared with L-Met–fed pigs in the present study could be due in the former to greater Cys production from body protein, lower Cys depletion, or both. The major Cys synthesis pathway is transsulfuration converting Hcy to Cys. Previously, we have shown that the transsulfuration rate was lower in pigs fed a Met-limited diet supplemented with

TABLE 2 Concentrations of selected amino acids, vitamins, oxidative stress markers, and antioxidant defense enzymes in plasma of pigs after 14-h overnight feed deprivation having been fed diets containing differing Met sources starting after weaning[1]

					P value[2]	
Parameter	Age, d	LMET	DLMET	DLHMTBA	Diet	Age	Diet × age
Met, μM	54	18 ± 2	16 ± 2	18 ± 2	0.582		
Cys, μM	54	88 ± 4	86 ± 4	87 ± 4	0.850		
Gly, mM	54	1.05 ± 0.7	1.06 ± 0.6	1.02 ± 0.7	0.931		
Gln, μM	54	632 ± 41	664 ± 40	657 ± 43	0.851		
Tau, μM	54	46 ± 2	41 ± 2	40 ± 2	0.164		
Vitamin A, nmol/L	57	490 ± 40	540 ± 40	560 ± 40	0.201		
Vitamin E, μg/L	57	121 ± 14	128 ± 14	117 ± 15	0.844		
TBARS, nmol/L	57	840 ± 12	530 ± 12	590 ± 13	0.284	0.708	0.404
	62	750 ± 13	760 ± 12	570 ± 14			
dROMs, mg H_2O_2/dL	57	25 ± 2	25 ± 2	24 ± 2	0.821	<0.001	0.554
	62	34 ± 2*	31 ± 2*	32 ± 2*			
8-iso-$PGF_{2\alpha}$, pg/mL	57	105 ± 14	150 ± 13	131 ± 16	0.871	0.548	0.487
	62	134 ± 15	116 ± 14	120 ± 17			
GPX, U/L	57	676 ± 53	596 ± 51	636 ± 56	0.183	<0.010	0.479
	62	740 ± 56	738 ± 54	841 ± 60*			
SOD, mU/mL	57	380 ± 20	360 ± 20	370 ± 20	0.392	<0.001	0.116
	62	270 ± 20*,+	300 ± 20*	330 ± 20			

[1] All values are least-square means ± SE. Met-deficient basal diet (69% of SID Met + Cys recommendation) supplemented with either L-Met (+0.15% LMET, $n = 9$); DL-Met (+0.15%; DLMET, $n = 9$–10), or DL-2-hydroxy-4-methylthiobutyric acid (+0.17%; DLHMTBA, $n = 8$–9). *Different relative to age 57 d, within column, $P < 0.05$, Tukey–Kramer test. +Tendency to differ compared with DLHMTBA, within row, $P < 0.1$, Tukey–Kramer test. dROM, reactive oxygen metabolite; GPX, glutathione peroxidase; SOD, superoxide dismutase; Tau, taurine; TBARS, thiobarbituric acid substance; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$.
[2] ANOVA *F* test.

DL-HMTBA relative to L- and DL-Met supplementation (17). The lower transsulfuration rate was accompanied by higher remethylation, whereas transmethylation was not different between DL-HMTBA– and Met–supplemented groups (17). Thus, the trend for greater Cys flux along with diminished transsulfuration in DLHMTBA compared with LMET pigs points to a lower Cys depletion.

As demonstrated by the $^{2}H_{2}$-Gly tracer experiment in overnight feed-deprived animals, the FSR of GSH was 49% lower and the total GSH concentration in RBCs 8–11% lower in pigs fed diets supplemented with DL-HMTBA and DL-Met compared with L-Met–supplemented pigs. The differences in the GSH variables could be due to different kinetics of postabsorptive processes. More specifically, the differences between DL-HMTBA and L-Met supplementation can be attributed to the lower transsulfuration detected in DLHMTBA than in LMET pigs, whereas transsulfuration was not different between DL-Met and L-Met supplementations (17). Ingestion of a sulfur AA–free diet diminished whole blood GSH synthesis rate (8), but consumption of a Met-adequate Cys-free diet did not limit erythrocyte GSH synthesis in young men (34). Supplementation of L-Met to a Met-deficient diet in weaned piglets increased plasma Cys and GSH concentrations, whereas the transport of Met across the jejunal mucosa did not differ among diets (35). In contrast, a study using a ^{13}C-Met tracer approach reported on the apparently higher net portal appearance of Met when 35-d-old barrows were fed a diet containing DL-HMTBA compared with DL-Met (14). However, these authors did not account for the different total Met intake levels in their study, which raises the question whether DL-HMTB feeding does indeed increase Met bioavailability. However, bioavailability of L-Met and DL-Met as assessed by average daily gain recordings as well as protein synthesis was reported to be comparable in weaned piglets (17, 32, 33).

Although RBCs are the predominant site in the circulation for the synthesis of GSH, a minor portion of circulatory GSH found in plasma originates from the liver (6). Key factors determining the hepatic GSH synthesis rate are the availability of the sulfur AA precursors and the activity of the rate-limiting enzyme GCL (6). The hepatic transcript abundance of GCL was higher in DLHMTBA pigs than in LMET pigs and was directly associated with the greater Cys flux of pigs ingesting the DL-HMTBA diet. However, gene expression of secondary enzymes controlling GSH synthesis (*GSS* and *GR*) as well as hepatic GSH concentration did not differ among dietary groups. Yet, the *GPX* mRNA abundance was higher in DLHMTBA pigs than in DLMET feed-deprived pigs, a result supporting the idea of a DL-HMTBA–induced Met deficiency because hepatic GPX activity and transcript amounts were also increased after dietary Met restriction in pigs (29). It seems that upregulation of transcripts coding for enzymes related to GSH synthesis can be interpreted as a compensatory mechanism counteracting a reduced hepatic GSH synthesis rate in DLHMTBA pigs; however, this assumption needs to be confirmed in a separate tracer study with repetitive liver samplings.

A change in the GSH synthesis rate in a tissue can also be due to differences in the immune status of the pigs (12). Under ad libitum feed consumption, the concentrations of antioxidants (GSH), antioxidant enzymes (GPX), and oxidation products (TBARS) in liver and jejunum were not different in pigs fed diets supplemented with L-Met or DL-Met (30). Likewise, concentrations of the plasma oxidative stress markers TBARS, dROMs, 8-iso-$PGF_{2\alpha}$, and GPX measured in the feed-deprived state also did not differ among LMET, DLMET, and DLHMTBA groups of the present study. These results suggest collectively that although the GSH FSR, GSH, and total GSH concentrations in RBCs differed between LMET and DLMET pigs, this difference did not affect protein accretion, growth, and antioxidant capacity under ad libitum feed consumption.

In conclusion, our results suggest that although the GSH FSR, GSH, and total GSH concentrations in RBCs were higher in pigs fed L-MET compared with DL-MET or DL-HMTBA diets after feed deprivation, oxidative stress and defense status were not affected by these dietary supplementations. Although the mechanism underlying the reduction in GSH FSR and RBC GSH concentrations in feed-deprived DL-Met pigs remains to be elucidated, the DL-HMTBA supplementation comprehensively showed reduced availability of Met for GSH synthesis and counterregulatory transcriptional responses in liver in an attempt to cope with Met deficiency and lower transsulfuration. Further studies are needed to determine the activity and transcription of genes involved in GSH synthesis and oxidative stress defense in the fed status and examine whether the antioxidant capacity of pigs is affected by the oxidative stress level under challenge conditions, for example, during inflammation or temperature stress.

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