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ORIGINAL ARTICLE

Effects of 2-hydroxy-4-methylthiobutyrate on portal plasma flow and net portal appearance of amino acids in piglets

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Abstract To determine whether portal plasma flow (PPF) and net portal appearance of amino acids (AA) could be affected by 2-hydroxy-4-methylthiobutyrate (HMB), six barrows (35-day-old, 8.6 ± 1.4 kg), implanted with arterial, portal and mesenteric catheters, were fed a DL-methionine (as the control) or HMB-supplemented diet once hourly and infused intramesenterically with 1% *p*-amino hippurate. PPF was numerically 9% higher ($P = 0.09$) in HMB-fed pigs than in controls over a 4–6 h period. Compared with controls, pigs fed the HMB diet had increased ($P < 0.05$) net portal balance and/or appearance of leucine, isoleucine, histidine, arginine and alanine, but had decreased ($P < 0.05$) portal appearance of glutamate over a 6-h period. The concentration of acetate in the lumen of the distal small intestine was higher ($P = 0.01$) in HMB-fed pigs than in controls (25.14 vs. 7.64 mmol/kg). mRNA levels for proglucagon and endothelial nitric-oxide synthase (eNOS) in stomach and proximal small intestine, and mRNA levels for GLP-2 receptor (GLP-2R) in stomach were higher ($P < 0.05$) in HMB-fed pigs compared with those in controls. Collectively, HMB supplementation increased concentrations of short-chain fatty acids in intestinal lumen, expression of proglucagon, GLP-2R, and eNOS genes, and net portal absorption of AA. These novel findings from the study with pigs may also have important implications for intestinal nutrition and health in humans.

Keywords Amino acids · Portal plasma flow · Proglucagon · Short-chain fatty acids · 2-Hydroxy-4-methylthiobutyrate

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Introduction

The primary fate of essential amino acids (AA) is presumably to protein synthesis; however, recent intriguing data have demonstrated that catabolism dominates the first-pass utilization of these AA by the gut (Stoll et al. 1998). The extensive catabolism of dietary essential AA by the intestine results in decreased absorption of dietary AA into the portal blood and represents a nutritional loss to the animals (Wu 1998). Therefore, increasing the net portal appearance of dietary AA may contribute to improved AA utilization efficiency (Wu 1998; Bertolo et al. 2005).

It is established that gut blood flow rates play an important role in affecting nutrient absorption (Topping and Clifton 2001; Guan et al. 2003). Previous studies showed that enteral supply of short chain fatty acids (SCFAs) could lead to substantially enhanced splanchnic blood flow (Kvietys and Granger 1981; Mortensen and Nielsen 1995) and increased secretion of glucagon-like peptide-2 (GLP-2), which is a product of proglucagon gene and demonstrated to have a vasodilatory effect (Tappenden

et al. 1998; Tappenden and McBurney 1998; Feltrin et al. 2006). Because greater blood flow was considered beneficial for enhancing transport of absorbed nutrients (Topping and Clifton 2001; Guan et al. 2003), we anticipated that portal nutrient absorption efficiency could be improved by dietary supplementation of SCFAs. 2-Hydroxy-4-methylthiobutyrate (HMB), a methionine precursor often added to swine and poultry diets (Knight et al. 1998), is a SCFA. Furthermore, there is evidence that gastrointestinal SCFA profile can be affected by dietary HMB supplementation (Noftsger et al. 2003). To our knowledge, however, few study reports are available on responses of blood flow and nutrient absorption to dietary HMB supplementation.

In this study, we hypothesized that HMB may increase portal blood flow and thus net portal appearance of AA. Therefore, piglets implanted with arterial and portal catheters were used as an animal model to investigate the association of portal AA absorption with portal blood flow following HMB supplementation. Additionally, gastrointestinal SCFA concentrations and mRNA levels for proglucagon, GLP-2 receptor (GLP-2R), and endothelial nitric oxide synthase (eNOS) were determined to explain how HMB would enhance portal blood flow.

Materials and methods

Animals and diets

The protocol of this study was approved by the Animal Care and Use Committee of College of Animal Sciences and Technology, Huazhong Agricultural University, and was carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Ten 21-day-old piglets from the same litter [6.3 ± 1.1 kg bodyweight (BW)], Large White × Landrace) were individually housed in metabolic cages (1.5 m × 0.75 m) located in the same air-conditioned room. Pigs were fed one of two diets (control vs. experimental diet) with five pigs per diet during the experimental period from 21 to 35 days of age. The two diets (Table 1), formulated to meet National Research Council (NRC 1998) nutrient requirements, had the same amounts of corn, dried whey, porcine plasma and dehulled soybean meal. The only difference between the two diets was that 30% of the total methionine in control and experimental diet was provided by DL-met and HMB, respectively. Pigs were offered the meal at a rate of 50 g feed kg BW^{-1} day^{-1}, which supplied 8.81 g crude protein kg^{-1} day^{-1} and 725 kJ gross energy kg^{-1} day^{-1}. Pigs had free access to water at all times. After 7 days of adaption period, all pigs received a surgery as described (Stoll et al. 1998). Briefly, after overnight food deprivation, pigs were surgically implanted with catheters in one carotid artery (Tygon tubing, 2.41 mm OD), the portal vein (polyethylene tubing, 1.78 mm OD), and the mesenteric vein (Silicone tubing, 1.02 mm OD) under isoflurane anesthesia and strict aseptic conditions. The catheters were filled with sterile saline containing heparin (2.0×10^5 U/l). All of the catheters were protected with gauze pads and secured with an elastic bandage. The animals received an intramuscular injection of analgestic (0.1 mg/kg butorphenol tartrate) and antibiotic (20 mg/kg Ampicillin Sodium) twice daily during the first 3 days postsurgery. After the surgery, the piglets were offered parenteral nutrition for 12–24 h and were then progressively returned to their proceeding level of dietary intake for ≥5 days before the experimental protocol. At a postnatal age of 35 days, six barrows in good health and with well-kept catheters were studied for a 7-h experimental period. Their body weight was 8.6 ± 1.4 kg.

Table 1 Composition of experimental diets (as-fed basis)

Ingredients		Amino acids	Amino acid concentrations g/kg protein[a]
Corn	68.13	Threonine	56
Dried whey	11.50	Tryptophan	16
Soybean oil	2.00	Leucine	84
Dehulled soybean meal	4.50	Lysine	84
Porcine plasma	10.00	Phenylalanine	52
Methionine premix[b]	0.14	Valine	58
Lysine HCl	0.45	Methionine	22
Threonine	0.10	Isoleucine	46
Tryptophan	0.01	Histidine	31
Isoleucine	0.17	Proline	52
Valine	0.05	Arginine	55
Limestone	1.02	Tyrosine	28
Dicalcium phosphate	1.14	Cysteine	28
Vitamin premix[c]	0.30	Alanine	60
Mineral premix[d]	0.49	Serine	53
Total	100	Glutamate plus glutamine	149
		Aspartate plus asparagine	85
		Glycine	41

[a] The crude-protein content was analyzed to be 176 g per kg diet

[b] Supplied 0.12% methionine activity at the expense of corn as DL-methionine (99%) or alimet (88%, DL-2-hydroxy-4-methylthiobutyrate)

[c] Provided per kg of diet: vitamin A, 13,500 IU; vitamin D3, 3,000 IU; vitamin E, 24 mg; menadione, 3 mg; thiamine, 3 mg; riboflavin, 7.5 mg; niacin, 30 mg; D-panthothenic acid, 15 mg; vitamin B6, 3 mg; vitamin B12, 22.5 μg; D-biotin, 120 μg; folic acid, 1.5 mg

[d] Provided per kg of diet: copper, 140 mg; iron, 150 mg; manganese, 50 mg; zinc, 140 mg; iodine, 0.8 mg; selenium, 0.4 mg. flavours, 200 mg; antioxidant, 100 mg

Infusion protocol and blood collection

The piglets were deprived of feed from 1800 to 0800 hours. At 0655 hours, a 1% solution of *p*-amino hippurate (*p*AH, sodium salt, diluted in sterile saline at pH 7.5) was infused continuously with a screw-driven syringe constant-infusion pump (WZS-50F6, Medical Instrument Corporation of Zhejiang University, China) into the mesentric catheters at a rate of 3.820 ml/min for 5 min and then 0.788 ml/min for 7 h (Yen and Killefer 1987). At ~0745 hours, base-line arterial and portal blood samples (10 ml) were withdrawn into heparined tubes. From 0800 to 1400 hours, pigs were offered meals at hourly intervals, and the meal was the equivalent of one 24th of the daily intake (45 g/kg BW). From 0900 to 1400 hours, arterial and portal blood samples were taken at hourly intervals, and all the blood samples were immediately placed on ice. Within 10 min of collection, the blood was centrifuged at 4°C and 2,550×*g* for 15 min to separate plasma from cells. An aliquot of plasma was refrigerated and assayed within 24 h for pAH concentration. Another aliquot was stored at −80°C until analyzed for AA concentration.

Collection of tissue and digesta samples

Immediately after completion of blood samples, pigs were killed with an arterial injection of sodium pentobarbital (50 mg/kg BW) and sodium phenytoin (5 mg/kg BW). Immediately after death, the abdomen was opened and samples were collected from the stomach and intestine as described (Tappenden et al. 1998). Briefly, the intestine was rapidly excised from the ligament of Treitz to the ileocecal valve, freed of its mesenteric fat, rinsed in ice-cold saline, and weighed. The intestine was divided into three segments with the most proximal and distal segments representing the jejunum and ileum, respectively. A 2-cm section of tissue was taken at a pre-determined distance from the two ends of the intestines, snap frozen in liquid nitrogen, and stored at −80°C for subsequent RNA isolation. Digesta samples in the stomach, distal small intestine and colon were also collected and refrigerated for subsequent analysis of SCFAs.

Sample analysis

pAH analysis

*p*AH in plasma samples were analyzed as described (Li et al. 2003). Briefly, a 1 ml of portal or arterial plasma sample was placed in a 50 ml Erlenmeyer flask, 2 ml of 0.025% (wt/v) sodium nitrite and 0.003% Brij-35 in 1.206 M HCl, 0.325% ammonium sulfamate solution containing 0.002% Brij-35, and 0.105% (*N*-α-naphthyl)ethylenediamine dihydrochloride in 3.430 M ethanol were ordinally added, and then de-ionized water was added to make the total volume approach 50 ml. After a 30-min period of incubation at 25°C, the absorbance at 550 nm was detected by VIS-7220 Spectrophotometer (Beijing Rayleigh Analytical Instrument Corporation, China). The *p*AH standard curve was generated by the addition of known concentrations of *p*AH into the complete reaction mixture, minus the plasma, and incubating these under reaction conditions for 30 min followed by the spectrophotometric assay described above. The portal plasma flow (PPF) rate (l kg^{-1} h^{-1}) was calculated as the following equation: $PPF = C_i \times IR \times [(pAH_p - pAH_a)\ BW]^{-1}$ where C_i is the concentration of infused *p*AH solution (g/l), IR is the infusion rate (l/h) of *p*AH, pAH_p and pAH_a are the concentration (g/l) of pAH detected in the portal vein and artery, respectively, and BW is the bodyweight (kg).

AA analysis

For AA analysis, the diet was hydrolyzed in 6 N HCl for 24 h at 110°C, and the frozen plasma samples were thawed at 4°C and deproteinized using 2.5 ml of 7.5% (wt/v) trichloroacetic acid per milliliter of plasma. For methionine and cysteine analysis, the dietary protein was oxidized with performic acid for 16 h at 4°C before hydrolysis in 6 N HCl for 24 h at 110°C. The AA concentration of deproteinized plasma and hydrolyzed protein was determined by ion-exchange chromatography with an L8800 high-speed amino acid analyser (Hitachi, Tokyo). Net portal mass balances (μmol kg^{-1} h^{-1}) of AA were calculated by the following formula: portal mass balance = $PPF \times (C_p - C_a)$ where PPF is the portal plasma flow rate (l kg^{-1} h^{-1}) and C_p and C_a are the portal and arterial plasma concentrations of AA (μmol/l). Fractional mass balance (percentage of intake) = portal balance × 100/intake.

SCFAs analysis

SCFAs including acetate, propionate and butyrate in digesta samples were analyzed as described (Wang 2006). Briefly, 2 g of digesta was weighed into a 5 ml centrifuge tube and added with 2 ml of de-ionized water. After the tube was capped, the content was vortex mixed for 30 min and then centrifuged (20,000×*g*, 4°C) for 20 min. Two milliliters of the supernatant was removed by aspiration into another 5 ml centrifuge tube, added with 400 μl of 25% metaphosphate, vortex mixed for 30 min and then left to stand overnight at 4°C. Next, the contents were centrifuged (20,000×*g*, 4°C) for 20 min and the clear supernatant was removed by aspiration for the following gas chromatography analysis. The samples were analyzed

by CP-3800 gas chromatography (Varian, Inc., USA) equipped with a 1177 injector, a flame ionization detector and a capillary chromatographic column (HP-INNOWAX, 30 m × 0.32 mm id × 0.25 μm). The injector temperature was 240°C, detector temperature was 300°C, column flux was 1.4 ml/min, hydrogen flux was 30 ml/min, and air flux was 400 ml/min. The temperature program was as follows: 70°C hold 6 min, increase to 140°C at 5°C/min, increase to 200°C at 50°C/min and hold 1 min. Peaks were identified by comparing their retention times with individual reference standard fatty acids.

mRNA abundance analysis

The relative mRNA abundance of proglucagon, GLP-2R and eNOS was determined by semiquantitative RT-PCR (Spencer and Christensen 1999). Total RNA was extracted from the snap-frozen samples using the TRIzol regent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's specifications. Concentration of RNA was quantified by measuring absorbance at 260 and 280 nm, and RNA integrity was verified by electrophoresis on a 1% (wt/v) agarose formaldehyde gel stained with ethidium bromide. Reverse transcription was performed by incubating 2 μg total RNA, 1 μl of random decamer primers (25 mM; Toyobo, Japan), 4 μl of oligo-$(dT)_{20}$ (10 mM; Toyobo, Japan), 0.5 mM each of the 4 dNTPs, 5× first-strand buffer, 40 U RNasin (Promega), 150 U M-MLV Reverse Transcriptase (Promega) in a final volume of 50 μl at 42°C for 1 h. PCR were performed in a 25 μl total volume containing 1 μl (proglucagon) or 3 μl (GLP-2R or eNOS) template cDNA, 0.5 mM dNTPs, 10× PCR buffer, 1.25 U *Taq* DNA polymerase (Toyobo, Japan), and specific sense and antisense oligonucleotides (Table 2) at 0.5 mM each. The samples for proglucagon, GLP-2R and eNOS genes, respectively, were denatured at 94, 95, 94°C for 4, 2, 4 min and underwent amplification cycles with denaturation at 94, 95, 94°C for 30, 60, 60 s, annealing for 30, 30, 30 s at 57, 54, 59°C, and extension for 30, 60, 60 s at 72, 72, 68°C. An additional extension at 72, 72, 68°C for 5, 10, 7 min was performed for proglucagon, GLP-2R and eNOS genes, respectively, and the samples were then cooled to 4°C. The linear amplification range was tested on the adjusted cDNA. The optimal cycle number was considered to be 2 cycles lower than the highest cycle of linearity. PCR products were electrophoresed on 2% (wt/v) agarose/Tris–borate EDTA gels, stained with ethidium bromide, and analyzed on a G:BOX-HR Gel Documentation System (Syngene, Cambridge, UK). mRNA abundance was expressed as band intensity relative to 18S rRNA band intensity. Data for each replicate represented the mean of three individual reverse transcription PCR.

Primers

Primer sequences and optimal PCR annealing temperatures were listed in Table 2. Oligonucleotides to recognize the pig proglucagon were designed from cDNA sequences in pigs (accession number AY242124). The sense and antisense oligonucleotides corresponded to nucleotides 204–222 and 418–437, respectively. Oligonucleotides to identify the pig GLP-2R were designed as described (Petersen et al. 2001). The sense and antisense oligonucleotides corresponded to nucleotides 529–548 and 964–983, respectively, in rat (accession number AF105368) and human (AF105367). Oligonucleotides to recognize the pig eNOS were designed as described (Fu et al. 2005). The sense and antisense oligonucleotides corresponded to nucleotides 3,211–3,231 and 3,531–3,552, respectively, in pigs (AY266137).

Statistics

Data were statistically analyzed as described (Steel and Torrie 1980). Differences in means of SCFA concentration and mRNA abundance between the control group and

Table 2 Oligonucleotide sense and antisense sequences, annealing temperatures, and cycle number

Oligonucleotides	Annealing temperatures and cycles	Sequences
Proglucagon	57°C, 29 cycles	
Sense		5′-CAGGCGTGCCCAGGATTTT-3′
Antisense		5′-TGTCTGCGGCGGAGTTCTTC-3′
GLP-2R	54°C, 31 cycles	
Sense		5′-ACCTTGCAGCTGATGTACAC-3′
Antisense		5′-GTGTTCTCCAGGTGTGCACG-3′
eNOS	59°C, 35 cycles	
Sense		5′-GTGTTTGGCCGAGTCCTCACC-3′
Antisense		5′-CTCCTGCAAGGAAAAGCTCTG-3′

HMB group, were analyzed by a two-sample unpaired *t* test. Differences in means of PPF between infusion periods for each group of pigs were analyzed by a paired *t* test. For comparisons of PPF, portal balance and appearance of AA between the control group and HMB group, a repeated-measure ANOVA (V8.1, SAS Institute Inc., Cary, NC, USA) analysis was performed with time of pAH infusion as the repeated-measure effect. *P* values ≤ 0.05 were considered statistical significance.

Results

Portal plasma flow (PPF)

The PPF of HMB-fed pigs and control pigs measured after overnight food deprivation (0 h) and for each hour after the start of the hourly feeding protocol is shown in Fig. 1. At the start of the study (0 h), there was no difference ($P > 0.10$) in PPF (1.71 vs. 1.67 l kg^{-1} h^{-1}) between HMB-fed pigs and controls. Within 1 h after the start of feeding, PPF was rapidly increased ($P < 0.05$) in both groups of pigs compared to their corresponding base-line PPF. PPF remained above baseline flow rates from 1 h throughout the entire study period, reaching a maximum at 6 h in both groups of pigs. PPF was numerically 9% higher ($P = 0.09$) in HMB-fed pigs than in controls that occurred 4 h after the start of feeding. The time or interaction between time and treatment for PPF over the 4–6 h period did not differ ($P > 0.05$). However, there was an effect ($P < 0.01$) of time on PPF rates over the 1–6 h period.

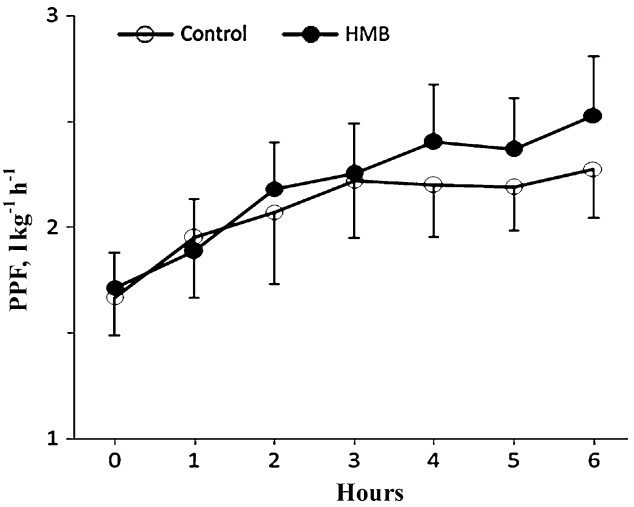

Fig. 1 Portal plasma flow (l kg^{-1} h^{-1}) in piglets in response to an hourly feeding of control or HMB diet. Values are mean ± SD, $n = 3$. Hours refer to the time after the initiation of feeding. Based on repeated-measure ANOVA, portal plasma flow was higher ($P < 0.05$) at $t = 1$–6 than at baseline ($t = 0$) in both groups

Net portal AA appearance

There were no differences ($P > 0.10$) in the net portal appearance of total essential AA, conditionally essential AA, nonessential AA or total AA over the 1–3 h feeding period (Table 3). However, the net portal balance and appearance of total conditionally essential AA in HMB-fed pigs were increased ($P < 0.05$) over the 4–6 h period compared with those in the control group, and also there was an increase ($P < 0.05$) in the net portal balance of total essential AA. Consequently, there was an increase ($P < 0.05$) in the net portal appearance of total conditionally essential AA over the 1–6 h period. Note that there was a numerical increase ($P = 0.10$) in the net portal appearance of total essential AA during the 6-h post-feeding. With the increase of time, the net portal balance and appearance of total essential AA and total AA were enhanced ($P < 0.01$) regardless of treatments over the 1–6 h period. For individual AA, the net portal balance (μmol kg^{-1} h^{-1}) of leucine (113 vs. 92), isoleucine (66 vs. 53) and histidine (52 vs. 38) among essential AA was increased ($P < 0.05$) following HMB supplementation over the 1–6 h period. Arginine is shown to be the single conditionally essential AA with enhanced ($P < 0.05$) net portal balance (100 vs. 79) and appearance (103 vs. 85%) following HMB supplementation. Although the portal balance or appearance of total nonessential AA was not different ($P > 0.10$) between groups, there was an increase ($P = 0.03$) in the net portal balance of alanine (356 vs. 279) in HMB-fed pigs compared with those in the control group. Interestingly, the net portal balance (29 vs. 105) and appearance (10 vs. 35%) of glutamate were decreased ($P < 0.001$) in HMB-fed pigs compared with those in the control group. The net portal methionine appearance was not different ($P = 0.18$) between HMB-fed pigs and controls over the 1–6 h period (59 vs. 51%).

SCFA concentration

There were no differences ($P > 0.10$) in the concentration of acetate, propionate or butyrate in the lumen of the stomach (Table 4). However, acetate concentration in the lumen of the distal small intestinal of HMB-fed pigs was three times ($P = 0.01$) that in the control group. Colonic propionate level was lower ($P = 0.01$) in HMB-fed pigs than in controls. There was a numerical decrease ($P = 0.06$) in colonic butyrate concentration in HMB-fed pigs compared with the control group.

mRNA abundance of proglucagon, GLP-2R and eNOS

Changes in mRNA abundance of proglucagon, GLP-2R and eNOS in the stomach, proximal and distal small

Table 3 Intake, portal mass balance and net portal appearance of total essential amino acids (AA), total conditionally essential AA, total nonessential AA and total AA in piglets fed once hourly with DL-methionine (DL-met) or 2-hydroxy-4-methylthiobutyrate (HMB)

	Intake	Portal balance	Portal appearance, % of intake		
	μmol kg^{-1} h^{-1}	μmol kg^{-1} h^{-1}	1–3 h	4–6 h	1–6 h
Total essential AA					
DL-Met	962 ± 26	541 ± 108	54 ± 12	59 ± 12	56 ± 12
HMB	1000 ± 115	618 ± 118	56 ± 13	69 ± 11	62 ± 14
P value	0.60	0.01	0.63	0.12	0.10
Total conditionally essential AA					
DL-Met	128 ± 3	89 ± 24	68 ± 23	69 ± 14	68 ± 18
HMB	133 ± 15	107 ± 23	76 ± 19	85 ± 18	81 ± 18
P value	0.60	0.02	0.10	0.04	0.01
Total nonessential AA					
DL-Met	1039 ± 28	620 ± 111	59 ± 12	60 ± 10	60 ± 11
HMB	1080 ± 124	649 ± 199	54 ± 18	68 ± 24	61 ± 22
P value	0.60	0.58	0.44	0.41	0.81
Total AA					
DL-Met	2129 ± 57	1250 ± 217	57 ± 12	60 ± 10	59 ± 11
HMB	2213 ± 255	1373 ± 296	56 ± 14	70 ± 16	63 ± 16
P value	0.60	0.09	0.85	0.19	0.32

Values are mean ± SD, $n = 3$. Essential AA include threonine, leucine, lysine, phenylalanine, valine, methionine, isoleucine and histidine. Conditionally essential AA include arginine and cysteine. Nonessential AA include tyrosine, alanine, glutamate, aspartate, serine and glycine

intestine of HMB-fed pigs compared to those of controls are presented in Fig. 2. Proglucagon mRNA abundance was increased ($P < 0.05$) in the stomach (1.78 vs. 0.38) and the proximal small intestine (1.42 vs. 0.55) of HMB-fed pigs, compared with the control group, but the values did not differ ($P > 0.10$) in the distal small intestine (3.35 vs. 3.03). GLP-2R mRNA abundance in the stomach (0.41 vs. 0.10) of HMB-fed pigs was higher ($P < 0.01$) than that in the control group, while there was no difference ($P > 0.10$) in the proximal intestine (0.56 vs. 0.47) or distal intestine (0.98 vs. 0.77) between the two groups of pigs. eNOS mRNA abundance in the stomach (1.15 vs. 0.24) and the proximal intestine (0.26 vs. 0.19) of HMB-fed pigs was also higher ($P < 0.05$) than that in control pigs, but no difference ($P > 0.10$) in the distal intestine (0.61 vs. 0.52) was observed between the two groups.

Table 4 The effect of 2-hydroxy-4-methylthiobutyrate (HMB) supplementation on short-chain fatty acid concentrations in the gastrointestinal tract of pigs

	Stomach	Distal small intestine	Colon
Acetate (mmol/kg)			
DL-Met	6.07 ± 1.25	7.64 ± 2.37	43.1 ± 2.76
HMB	5.34 ± 0.76	25.1 ± 0.49	36.7 ± 7.05
P value	0.44	0.01	0.32
Propionate (mmol/kg)			
DL-Met	0.14 ± 0.02	0.45 ± 0.09	18.4 ± 0.72
HMB	0.12 ± 0.04	0.62 ± 0.09	13.1 ± 1.22
P value	0.38	0.19	0.01
Butyrate (mmol/kg)			
DL-Met	0.030 ± 0.017	0.137 ± 0.002	5.60 ± 0.44
HMB	0.017 ± 0.004	0.149 ± 0.009	2.79 ± 1.25
P value	0.36	0.21	0.06

Values are mean ± SD, $n = 3$

Discussion

A major goal of the present study was to determine whether portal blood flow and net portal AA appearance were responsive to dietary HMB supplementation. Given evidence that enteral feeding and intake is a well-established stimulus of small intestinal blood flow (Fara and Madden 1975; Gladman et al. 1991), an hourly feeding regimen was performed to make sure that all pigs have an equal feed intake (expressed on a per-unit BW basis) and to have a good estimate of the actual feed intake during the infusion. The results indicated that PPF was increased rapidly within 1 h after the start of feeding, reaching a maximum at 6 h, which agreed well with previous reports (van Goudoever et al. 2001). Interestingly, HMB numerically increased PPF by 9% over the 4–6 h feeding period. Notably, the net portal balance and appearance of total conditionally essential AA were enhanced in HMB-fed pigs compared with those in the control group over the 4- to 6-h period. For individual AA, there was marked increase in the net portal balance and/or appearance of leucine, isoleucine, histidine, arginine and alanine in HMB-fed pigs compared with those in the control group over the 4- to 6-h period. In contrast, neither PPF rates nor net portal AA appearance differed between HMB-fed pigs and controls over the 1–3 h period. Taken together, we suggest that the enhanced PPF

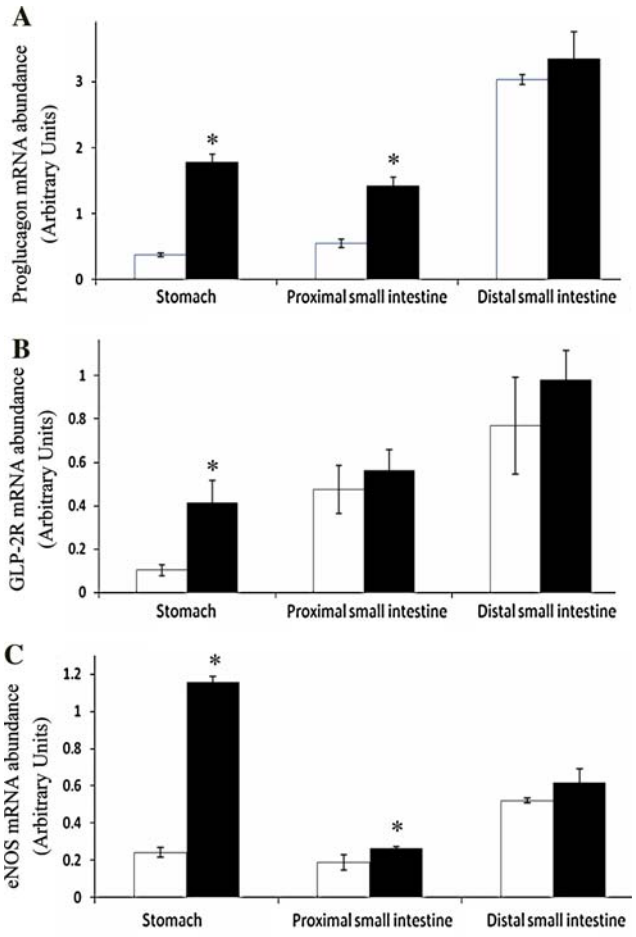

Fig. 2 Relative mRNA abundance of proglucagon (**a**), GLP-2R (**b**) and eNOS (**c**) in the stomach, proximal and distal small intestine of HMB-fed pigs ($n = 3$, *solid bars*) compared with that of controls ($n = 3$, *open bars*). mRNA abundance, measured by semiquantitative RT-PCR, is expressed relative to 18S rRNA. *Significant difference between treatments ($P < 0.05$)

might contribute to improved net portal AA absorption, which is consistent with the previous report (Guan et al. 2003). Regardless of treatments, the consistent increase in PPF rates and the net portal appearance of total essential AA and total AA with the increase of time further support this view.

Given that the pattern of AA in the diet differs markedly from that in portal venous blood and does not reflect their availability to extraintestinal tissues (Stoll et al. 1998; Li et al. 2008), the net portal balance and appearance of individual AA may have an important nutritional implication. The lower net portal appearance of glutamate in HMB-fed pigs, which means that higher proportion of glutamate is metabolized by the intestine in the first-pass, coupled with the higher portal balance and/or appearance of leucine, isoleucine and histidine, suggests that the increase in glutamate catabolism may reduce the catabolism of dietary essential AA as energy substrates for the intestinal mucosa. Of particular interest, branched-chain AA and conditionally essential AA (arginine, proline, and glutamine) are now known to be extensively catabolized by enterocytes of growing pigs (Chen et al. 2007; Wu 1995, 1997). The increased net portal appearance of alanine in HMB-fed pigs may provide further evidence for the substantial catabolism of glutamate because alanine is an important nitrogenous product of the intestinal catabolism of glutamate (Windmueller 1982). Because systemic SCFAs and GLP-2 can up-regulate mucosa transport of arginine and glycine (Kato et al. 1999), the enhanced portal balance and/or appearance of arginine and glycine in HMB-fed pigs may be associated with the modulation of SCFA profile and GLP-2 secretion. Alternatively, arginine is synthesized from glutamine/glutamate and proline in pig enterocytes (Wu 1997; Wu and Morris 1998). Whether GLP-2 can stimulate intestinal arginine synthesis remains to be defined.

Another objective of the current work was to ascertain whether enhancement of portal blood flow may be associated with alterations of the gastrointestinal SCFA profile in HMB-supplemented pigs. HMB is absorbed rapidly and in large quantities into the upper gastrointestinal cells after feeding (Richards et al. 2005). Furthermore, there is increasing evidence that SCFAs affect structural and functional aspects of intestinal adaptation via up-regulation of GLP-2, a product of the proglucagon gene (Tappenden et al. 2003). Consistent with this concept, proglucagon mRNA abundance was increased in HMB-fed pigs compared with that in the control group. This result suggests increased secretion of GLP-2 (Tappenden et al. 1998; Tappenden and McBurney 1998; Feltrin et al. 2006). It is noteworthy that GLP-2 acutely increased blood flow in the proximal small intestine (Stephens et al. 2006). Therefore, the acute expression of proglucagon and the rapid secretion of GLP-2 following infusion of SCFAs (Tappenden et al. 1998; Tappenden and McBurney 1998; Feltrin et al. 2006) raised the possibility that PPF rates would respond to dietary HMB within 1–3 h postprandial. However, results of the present study indicate that the increase in PPF occurred within 4–6 h after feeding in young pigs. Additionally, the much higher concentration of acetate in the distal small intestine in HMB-fed pigs than in controls suggested that acetate may play an important role in up-regulating PPF. Because physiologic concentrations of SCFAs, particularly acetate, influenced intestinal blood flow (Kvietys and Granger 1981) and the increase in PPF occurred within a 4–6 h period rather than within a 1–3 h period, it is likely acetate rather than HMB that may result in increased blood flow.

Available evidence shows that the rectal infusion of SCFAs could lead to a greater splanchnic blood flow (Kvietys and Granger 1981), but little information is available about the mechanism of action of SCFAs.

Because SCFAs are potent stimulators of GLP-2 secretion in rodents and human subjects (Xiao et al. 1999; Thulesen et al. 1999), attempts were made to investigate a relationship between SCFA-enhanced blood flow and GLP-2 concentration. Previous studies have established that the actions of GLP-2 are mediated via its receptor, GLP-2R (Munroe et al. 1999). In addition, recent findings reveal that the GLP-2-mediated increase in portal blood flow is mainly associated with increased intestinal constitutive NOS activity and eNOS protein abundance (Guan et al. 2003). Considering that there is generally a correlation between mRNA and protein abundance (Greenbaum et al. 2003), we examined mRNA levels for proglucagon, GLP-2R and eNOS, which may provide further explanation for the mechanism by which HMB enhanced portal blood flow. Consistent with previous reports (Munroe et al. 1999), both proglucagon and GLP-2R were expressed in the stomach, jejunum and ileum independent of treatments. The consistent increase in mRNA abundance of proglucagon, GLP-2R and eNOS in the upper gastrointestinal tract following HMB supplementation suggests that the vasodilatory effects of SCFAs may be mediated by the enhanced secretion of GLP-2. Although plasma GLP-2 was not determined directly in the current study, increased GLP-2 secretion is associated with enhanced proglucagon expression (Tappenden et al. 1998; Tappenden and McBurney 1998; Feltrin et al. 2006). In contrast, the unchanged expression of proglucagon, GLP-2R and eNOS in the distal small intestine further supports the view that jejunum is the principal target for GLP-2 action (Drucker et al. 1996).

In summary, results of the present work support the hypothesis that dietary HMB may up-regulate portal blood flow and net portal absorption of AA. These effects of HMB were associated with increases in both concentrations of intestinal SCFAs and expression of proglucagon, GLP-2R and eNOS genes. The enhanced portal appearance of dietary AA might imply an additional nutritional significance of HMB in swine growth. These novel findings may also have important implications for intestinal nutrition and health in humans.

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