Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 1121–1127

Journal of Nutritional Biochemistry

Intrauterine growth restriction leads to changes in sulfur amino acid metabolism, but not global DNA methylation, in Yucatan miniature piglets☆

Dylan S. MacKay, Julie D. Brophy, Laura E. McBreairty, Ross A. McGowan, Robert F. Bertolo*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3X9

Received 31 August 2010; received in revised form 31 May 2011; accepted 23 June 2011

Abstract

Intrauterine growth restriction (IUGR), in both animals and humans, has been linked to metabolic syndrome later in life. There has been recent evidence that perturbations in sulfur amino acid metabolism may be involved in this early programming phenomenon. Methionine is the precursor for cellular methylation reactions and for the synthesis of cysteine. It has been suggested that the mechanism behind the "fetal origins" of adult diseases may be epigenetic, involving DNA methylation. Because we have recently demonstrated the fetal origins phenomenon in Yucatan miniature swine, we hypothesized that sulfur amino acid metabolism is altered in IUGR piglets. In this study, metabolites and the activities of sulfur amino acid cycle enzymes were analyzed in liver samples of 3- to 5-day-old runt (IUGR: 0.85±0.13 kg) and large (1.36±0.21 kg) Yucatan miniature pig littermates (n=6 pairs). The IUGR piglets had significantly lower specific and total activities of betaine-homocysteine methyltransferase (BHMT) and cystathionine γ-lyase (CGL) than larger littermates (P<.05). Expression of CGL (but not BHMT) mRNA was also lower in IUGR piglets (P<.05). This low CGL reduced cysteine and taurine concentrations in IUGR pigs and led to an accumulation of hepatic cystathionine, with lower homocysteine concentrations. Methylation index and liver global DNA methylation were unaltered. Reduced prenatal growth in Yucatan miniature piglets impairs their remethylation capacity as well as their ability to remove cystathionine and synthesize cysteine and taurine, which could have important implications on long-term health outcomes of IUGR neonates.

Keywords: Methionine; Enzyme activity; Homocysteine; Epigenetics; Early origins of adult diseases

1. Introduction

The "fetal origins" of adult disease hypothesizes that factors *in utero* can affect the development of disease later in life [1]. Many studies in both animals and humans have demonstrated links between prenatal growth rate and adult diseases, including obesity, cardiovascular diseases and type 2 diabetes [2]. Because the initial hypotheses generated from epidemiological data implicated small birth weight, the most successful animal model in fetal programming has been the protein-deficient rat dam [3]. The intrauterine growth-restricted (IUGR) rat pups became adult rats that exhibited symptoms of various chronic diseases such as obesity, coronary heart disease, hypertension, glucose intolerance, appetite dysregulation and osteoporosis [3]. In addition, postnatal catch-up growth in growth-retarded pups also led to reduced longevity and increased hypertension, similar to epidemiological data [4]. Recently, the protein-deficient rat dams were found to be hyperhomocysteinemic because the low-protein diet was disproportionately high in methionine and low in cysteine (Fig. 1) [5–7]. Hyperhomocysteinemia in pregnancy has been linked to intrauterine growth retardation [8], and hyperhomocysteinemia in children has been linked to development of obesity and hypertension [9]. Because the mediating mechanism of fetal programming does not seem to be protein deficiency per se, focus has been more recently directed toward imbalance of sulfur amino acid metabolism as a potential mechanism for this phenomenon. Indeed, Rees [7] concluded that changes in cell function only occur when the metabolism of sulfur amino acids is disturbed.

Methionine is required for protein synthesis and for oxidation to synthesize cysteine; the oxidation to cysteine involves both transmethylation and transsulfuration pathways (Fig. 1). However, the oxidative enzymes in the transsulfuration pathway are low *in utero* and may be limiting for cysteine synthesis [10,11]. With intrauterine growth retardation, where nutrient flow to the fetus is reduced, oxidative pathways are further reduced due to less substrate

Abbreviations: BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; IUGR, intrauterine growth-restricted.

☆ Grants, sponsors and funding sources: This work was supported in part by grants to R.F.B from the Canadian Institutes of Health Research and from the International Life Sciences Institute North America (ILSI N.A.) [for further information about ILSI N.A., call (202) 659-0074 or email ilsina@ilsi.org]. The opinions expressed herein are those of the authors and do not necessarily represent the views of ILSI N.A.

* Corresponding author. Tel.: +1 709 864 7954; fax: +1 709 864 2422.
E-mail address: rbertolo@mun.ca (R.F. Bertolo).

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.06.005

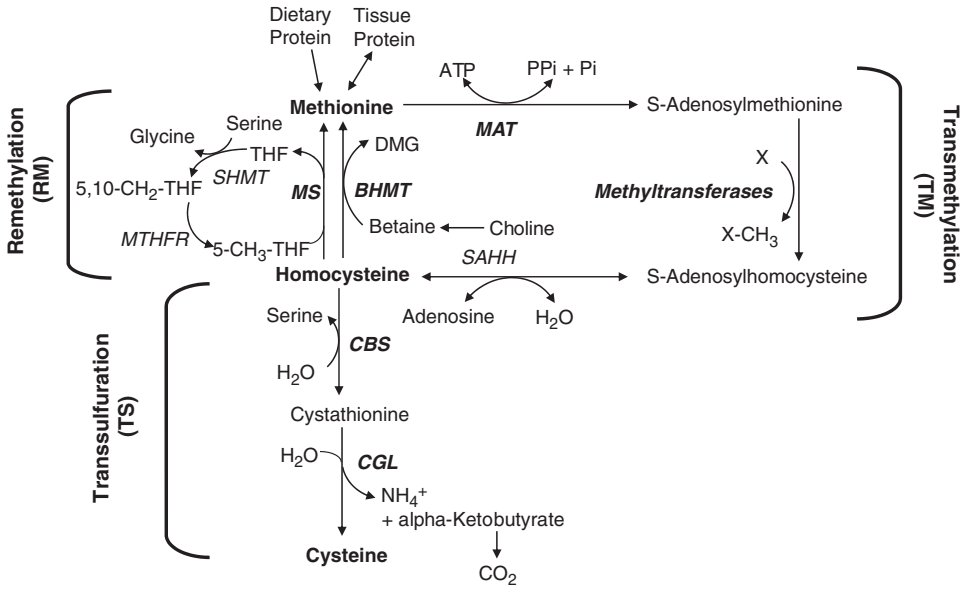

Fig. 1. Schematic of pathways in sulfur amino acid metabolism. Abbreviations: SAHH, *S*-adenosyl homocysteine hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; DMG, dimethylglycine.

availability. In the postnatal situation, compensatory growth following IUGR involves more efficient use of nutrients that, for amino acids, usually also involves a down-regulation of oxidative pathways [12]. So IUGR neonates have an imbalanced methionine metabolism such that oxidation, or transsulfuration, is reduced, leading to a potential accumulation of homocysteine.

One of the key transmethylation reactions is methylation of DNA, which is an inheritable mechanism regulating gene expression [13]. Indeed, such epigenetic changes have been proposed as the underlying mechanisms in the plasticity associated with early development that can plausibly program risk for disease in later life [13,14]. Methylation of DNA can be affected by dietary levels of methyl-donor components, such as methionine, choline and folate [13,15]. Moreover, in fetuses of rat dams fed methionine-imbalanced low-protein diets, global methylation of DNA was increased in several tissues, including the fetal liver [6]. Thus, changes in sulfur amino acid metabolism have been suggested as a possible mechanism by which environmental influences during fetal development could permanently affect fetal nutrition [7].

More recently, we [16,17] and others [18,19] have validated the pig as a model for early programming by demonstrating that the naturally occurring runt IUGR pig develops biomarkers for hypertension, diabetes, obesity and dyslipidemia early in adulthood compared to larger siblings. The pig has distinct advantages over rodent models of this phenomenon in that postnatal metabolism can be studied and the nutritional requirements of the pig are more similar to those of the human, especially with respect to amino acids [20]. Therefore, using the Yucatan miniature piglet as a model, we hypothesized that the lowered fetal nutrition that caused the subsequent low birth weight of the runt would also result in a decrease in the activity of the enzymes that facilitate the removal of excess sulfur amino acids, such as methionine and homocysteine. The imbalanced metabolic pathways and accumulation of homocysteine would also modify the methylation of DNA, thereby affecting the long-term regulation of gene expression and perhaps explaining the higher risk for chronic diseases later in life observed in IUGR piglets [16–19]. The main objectives of this study are (a) to determine if IUGR limits the capacity of certain sulfur amino acid enzymes, (b) to determine if IUGR affects expression of altered sulfur amino acid enzymes and (c) to determine if IUGR affects global methylation of DNA as a result of sulfur amino acid enzyme alterations.

2. Methods and materials

2.1. Reagents

L-[3-^{14}C]serine was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), and 5-[^{14}C]methyl-tetrahydrofolic acid barium salt was from Amersham Biosciences UK Limited (Buckinghamshire, UK). L-[1-^{14}C]methionine and *N,N,N*-trimethyl[methyl-^{14}C]glycine (^{14}C-betaine) were acquired from Moravek Biochemicals (Brea, CA, USA). The poly-prep prefilled chromatography columns (AG 1-X8, AG1-X4 and AG50W-X4 resins, 200–400 mesh, 0.8×4 cm) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other chemicals were of analytical grade and were from Sigma (St. Louis, MO, USA) or Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Animals

Yucatan miniature piglets (n=12) were obtained from the Memorial University of Newfoundland breeding colony. Animal care and handling procedures were approved by the Institutional Animal Care Committee and in accordance with the guidelines of the Canadian Council on Animal Care. Six pairs of littermates, each consisting of a spontaneously occurring runt (three males, three females) and its largest littermate (two males, four females), were removed from sows at 3–5 days of age. For the purposes of this study, a runt (i.e., IUGR) was defined as the smallest piglet in a litter that was at least 25% smaller than its largest littermate at birth. In this herd of Yucatan pigs, piglets fitting these parameters occur naturally in ~90% of litters. Within 1 h of removal from the sow, pigs were anesthetized with halothane, and blood was sampled via cardiac puncture and centrifuged immediately at 1500*g* for 10 min at 4°C to isolate plasma; given that pigs were within 1.5–2 h of last suckling, piglets were in fed state. Organs (liver, kidneys, colon, small intestine, lung and heart) were removed quickly under anesthesia and weighed, and samples were immediately frozen in liquid nitrogen and stored at −80°C until further analyses.

2.3. Liver homogenate preparation

Approximately 0.5–1.5 g of frozen liver was weighed and kept on ice. A homogenate of liver and 50 mM potassium phosphate dibasic (pH 7.0) buffer (1:5) was freshly prepared on ice using a Polytron homogenizer (Brinkmann Instruments, Mississauga, ON, Canada) for 30 s at 50% output. The homogenate was centrifuged at 20 000*g* for 30 min at 4°C (Beckman L8-M Ultracentrifuge), and the supernatant was removed and used immediately to measure the activities of sulfur amino acid metabolism enzymes. Homogenates were centrifuged at 13 000*g* for 5 min at 4°C. Protein concentration of homogenates was determined using the Biuret assay using porcine serum albumin as a standard.

2.4. Metabolite analyses

Total homocysteine and cysteine concentrations [21], *S*-adenosyl-L-methionine (SAM) and *S*-adenosyl-L-homocysteine (SAH) concentrations [22] and cystathionine concentrations [23] were determined by high-performance liquid chromatography (HPLC); all samples were first reduced with tris(2-carboxyethyl)phosphine. Other amino acids were determined by reverse-phase HPLC using phenylisothiocyanate derivatives [24]. Plasma urea concentrations were determined using a spectrophotometric assay kit (Sigma Chemical Co., St. Louis, MO, USA).

2.5. Enzyme assays

We measured the activities of three enzymes involved in the remethylation of homocysteine to methionine — methionine synthase (MS), betaine-homocysteine methyltransferase (BHMT) and methylenetetrahydrofolate reductase (MTHFR) — and three enzymes involved in catabolism of methionine to cysteine — methionine adenosyltransferase (MAT), cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). All assays were first demonstrated to be linear with respect to time (0–60 min) and protein concentration (0–2 mg). Specific activities were expressed as nmol of product per minute per mg protein. The hepatic enzyme capacity (nmol of product/min/kg body weight) was determined for each animal by multiplying the enzyme specific activity by the total protein content in the whole liver (i.e., mg protein/g wet liver×total liver weight) and correcting for body weight. Protein content of the liver was determined using a combustion method (FP-528; LECO Instruments, Mississauga, ON, Canada) and analyzing for total elemental nitrogen; a factor of 6.25 g protein/g nitrogen was used to convert nitrogen to protein.

Hepatic transglutaminase activity was measured using a colorimetric microassay as per kit instructions (Covalab UK Ltd., Cambridge, UK).

2.5.1. Methionine synthase

Methionine synthase was measured using an assay described by Koblin et al. [25]. Briefly, liver homogenate (100 μl of 2 mg/ml) was mixed with 100 μl substrate mixture [20 μM cyanocobalamin, 58 mM dithiothreitol (DTT), 0.5 mM SAM, 15 mM DL-homocysteine, 14 mM β-mercaptoethanol, 1 mM methyltetrahydrofolate with 2.5 μCi of 5-[^{14}C]methyl-tetrahydrofolic acid, and 175 mM potassium phosphate buffer, pH 7.5], capped with nitrogen and incubated in the dark at 37°C for 30 min. The reaction was stopped with 400 μl cold deionized water and stored on ice. The product, ^{14}C-methionine, was separated from the reactant, 5-[^{14}C]methyl-tetrahydrofolic acid, by ion-exchange column (AG 1-X8 resin, 200–400 mesh) and counted for radioactivity.

2.5.2. Betaine-homocysteine methyltransferase

Betaine-homocysteine methyltransferase activity was measured with an assay described by Garrow [26]. Briefly, liver homogenate (100 μl of 15 mg/ml) was added to the assay mixture (5 mM DL-homocysteine, 2 mM betaine with 0.1 μCi of ^{14}C-betaine, and 50 mM Tris, pH 7.5) and incubated at 37°C for 20 min; the reaction was stopped with 2.5 ml of cold deionized water and stored on ice. The samples (2 ml) were then applied to columns. The unreacted betaine was washed from a column (Dowex AG1-X4, 200–400 mesh, hydroxide form) with deionized water and, radiolabeled dimethylglycine and methionine were eluted with 3 ml of 1.5 M HCl and counted for radioactivity.

2.5.3. Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase activity was measured using the assay described by Engbersen et al. [27]. Briefly, liver homogenate (100 μl of 5 mg/ml) was added to 400 μl of assay mixture [0.27 M potassium phosphate, 1.72 mM EDTA, 17.25 mM ascorbic acid, 81 μM FAD, 21 mM menadione, 30 μM 5-[^{14}C]methyl-tetrahydrofolic acid, pH 6.8] and incubated in the dark at 37°C for 20 min; the reaction was stopped with 100 μl 3 M potassium acetate (pH 4.5), 200 μl dimedone (50 μmol dimedone in 200 μl 1:1 ethanol:water) and 10 μl of 1 M formaldehyde and then immediately placed in 95°C water bath for 15 min and cooled on ice for 10 min. After addition of 3 ml of toluene, samples were vortexed and centrifuged at low speed, and the toluene phase was measured for radioactivity.

2.5.4. Methionine adenosyltransferase

Methionine adenosyltransferase activity was assayed using the method of Duce et al. [28]. Briefly, 30 μl of homogenate (20 mg/ml) was added to 200 μl of assay mixture (125 mM Tris-HCl, 250 mM KCl, 12.5 mM $MgCl_2$, 1.25 mM DTT and 6.25 mM ATP, pH 7.80) and equilibrated at 37°C. Reaction was started by adding 20 μl of 62.5 mM ^{14}C-methionine and incubated in the dark at 37°C for 45 min. Reaction was stopped with 750 μl of cold water and stored on ice. Labeled product was eluted on columns (Biorad 50W-X4 resin, 200–400 mesh, NH_4 form) with 3 M NH_4OH and measured for radioactivity.

2.5.5. Cystathionine β-synthase

The CBS assay was performed as described by Taoka et al. [29]. Briefly, liver homogenate (150 μl of 15 mg/ml) was combined with 400 μl of substrate mixture (0.219 mM L-cystathionine, 62.5 mM DL-homocysteine, 3.125 mM DL-propargly glycine, 0.38 mM SAM and 0.625 mM pyridoxal-5-phosphate in 187.5 mM Tris-HCl/3.125 mM EDTA, pH 8.3) and allowed to equilibrate at 37°C. Fifty microliters of 300 mM ^{14}C-serine (0.1 μCi) was added to start the reaction, and after 30 min, the reaction was stopped with 300 μl of cold 15% trichloroacetic acid, and the samples were stored on ice. After centrifugation at 9000*g* for 10 min, 500 μl of the deproteinized sample was loaded on a column (AG 50W-X4 resin, 200–400 mesh, hydrogen form), and serine was washed out with deionized water, 1 M hydrochloric acid, followed again by deionized water. ^{14}C-cystathionine, was eluted with 5 ml of 3 M NaOH, and radioactivity was measured. This assay was light sensitive, and all sample tubes were covered in aluminum foil.

2.5.6. Cystathionine γ-lyase

Cystathionine γ-lyase was assayed as described by Stipanuk [30]. Briefly, 20% liver homogenate was incubated with 100 mM potassium phosphate buffer, 4.0 mM L-cystathionine, 0.125 mM pyridoxal 5′-phosphate, 0.32 mM NADH and 1.5 units of lactate dehydrogenase, pH 7.5. The decrease in absorbance at 340 nm was monitored to determine activity.

2.6. Real-time reverse transcriptase polymerase chain reaction analysis of BHMT and CGL gene expression

Total RNA was obtained from liver samples by the RNeasy kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using 1 μg of total RNA using QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA), including a genomic DNA wipeout step. Polymerase chain reaction (PCR) primers and probes for porcine BHMT (U53421) (forward primer, GTT CGC CAG CTT CAT C; reverse primer, CTC CAG CTT GTC CTC A; probe, CTC AGA GCC GGA TCG AAT GTC ATG C), CGL or cystathionase (DQ499449) (forward primer, CTG AGA GTT TGG GAG GAT A; reverse primer, CTT AGG CAC AGA TGA ATG A; probe, AAG TCT TGC TGA GCT TCC GGC A) and β-actin (DQ845171) (forward primer, CCC AGC ACG ATG AAG A; reverse primer, CGA TCC ACA CGG AGT A; probe, TCA AGA TCA TCG CGC CTC CAG A) were designed using RealTimeDesign software (Biosearch Technologies, Novato, CA, USA). Expression of β-actin was used as an endogenous reference to account for differences in sample loading and PCR efficiencies between reactions. Taqman probes for β-actin were labeled with reporter dye FAM on the 5′ end and quencher BHQ-1 on the 3′ end. Taqman probes for BHMT and CGL were labeled with reporter dye PULSAR 650 on the 3′ end and quencher BHQ-2 on the 5′ end. Amplification reactions were carried out using QuantiTect Multiplex PCR NoRox kit (Qiagen, Valencia, CA, USA) for a 20-μl duplex PCR volume. All reactions were performed in triplicate on a LightCycler 1.2 real-time PCR system (Roche, Laval, QC, Canada).The thermal cycling conditions were 15 min at 95°C to activate HotStarTaq DNA polymerase, followed by 40 cycles of 96°C for 1 min and 55°C for 1 min. A sample of reverse transcriptase reaction without reverse transcriptase enzyme and a sample of PCR mixture without cDNA were used as negative controls. Relative gene expression was calculated using a formula outlined by M.W. Pfaffl [31]; a PCR efficiency of 2.0 was used for all calculations.

2.7. Cytosine extension assay

Global methylation was estimated using the method of Pogribny et al. [32]. This method utilizes *Hpa*II, a methyl-sensitive endonuclease that cuts at the sequence CCGG, if the middle CG is unmethylated, leaving an overhanging guanine nucleotide. This guanine is paired with a radiolabeled cytosine using Taq polymerase, thereby estimating the amount of cleaved CCGG sequences in a DNA sample. Briefly, approximately 2 μg of genomic DNA is digested overnight with a fivefold excess of *Hpa*II, according to manufacturer's protocol (NEB, Ipswich, MA, USA). A 2-μg sample of genomic DNA in digestion buffer without endonuclease serves as a background control. A single nucleotide extension reaction was performed on each digested and undigested DNA sample in 25-μl reaction mixture containing 2 μg digested DNA, 2.5 μl 10× Native Taq buffer (Invitrogen, Burlington, ON, Canada), 0.75 μl 50 mM $MgCl^2$, 0.2 μl of Native Taq polymerase and 0.2 μl [^{3}H] CTP (57.4 Ci/mmol) (Moravek Radiochemical, Brea, CA, USA) and incubated at 55°C for 1 h. Ten microliters of each reaction digest was applied to two separate D-81 ion exchange filters, and each filter was washed three times in sodium phosphate buffer (pH 7.0). The filters were dried overnight and counted for radioactivity; results were expressed as relative [^{3}H]-dCTP incorporation (DPM)/0.5 μg of digested DNA.

2.8. Statistical analyses

All data are shown as mean±S.D., and differences were considered significant at $P<.05$. Comparisons were made using general linear model (Minitab Software version 15.1; Minitab Inc., State College, PA, USA) with each runt–large littermate representing a matching pair. Data were analyzed using a model containing runt/large, pairing, sex and sex×runt/large interaction; nonsignificant parameters were removed (never runt/large), and the model was reduced until the adjusted R^2 was maximized. Analyses in which sex was a significant covariate for runt/large differences are indicated; all other differences were in reduced models in which runt/large and pairing were maintained. Linear regressions were performed on data versus body weight (GraphPad Prism 5, GraphPad Software Inc.).To determine whether difference in pair's birth

Table 1
Body weight (BWT) and organ measurements in large and IUGR littermate piglets

		Large	IUGR
BWT	(kg)	1.36±0.21*	0.85±0.13
Liver	(g)	49.6±9.2*	32.1±4.5
	(g/kg BWT)	36.4±2.9	37.9±2.0
Kidney	(g)	10.7±2.4*	6.4±0.8
	(g/kg BWT)	7.8±0.7	7.7±1.0
Colon	(g)	19.7±5.5*	13.9±2.9
	(g/kg BWT)	14.7±4.3	16.9±5.0
Small intestine	(g)	56.0±11.7*	37.2±7.5
	(g/kg BWT)	40.9±2.7	43.6±2.9
	(cm)	455±51*	362±40
	(cm/kg BWT)	336±25	429±27*

Results are mean±S.D. (n=6 per group).
* P<.05.

weight was related to outcome difference, linear regressions were performed on significant outcomes, but no significant associations were found.

3. Results

3.1. Animals

At birth, IUGR piglets (0.665±0.121 kg; range: 0.460–0.807 kg) were 62%±12% (range, 45%–74%) of their respective large littermates (1.091±0.158 kg; range: 0.929–1.332 kg) (P<.001) and came from litters with 7.0±1.4 piglets (range: 5–9 per litter). At necropsy, IUGR pigs had body weights 63%±11% of their siblings and had proportionately smaller (P<.01) organs than littermates (liver: 66%±14%; kidneys: 62%±9%; colon: 72%±12%; small intestine mass: 68%±15%; small intestine length: 80%±11%) such that when organ measurements were corrected for body weight, no differences were observed for any organs between IUGR and littermates (Table 1); these data suggest no sparing effect for organ development during IUGR.

3.2. Metabolite concentrations

Plasma concentrations of urea and amino acids are displayed in Table 2. Of the sulfur metabolism amino acids, only plasma cysteine concentrations were significantly lower in IUGR versus large piglets (P=.03). Plasma concentrations of histidine, isoleucine, alanine, glutamate and hydroxyproline were significantly higher in IUGR piglets (P<.05); sex was a significant covariate for the plasma concentrations of histidine and isoleucine. Liver concentrations of taurine (P=.01) and homocysteine (P=.04) were lower, but cystathionine concentrations were 35% higher (P=.001) in IUGR piglets (Table 3); sex was a significant covariate for hepatic cystathionine and homocysteine concentrations.

3.3. Hepatic enzyme activities

Sulfur amino acid enzyme specific activities in piglet livers are displayed in Table 4. Compared to large littermates, IUGR livers had lower activities of the remethylation enzyme BHMT (P=.012) as well as the transsulfuration enzyme CGL (P=.04). Betaine-homocysteine methyltransferase (P=.07) and CGL (P=.22) only tended to correlate with body weight. Total hepatic enzyme capacities (Table 5) were lower in IUGR for BHMT (P=.006) and CGL (P=.014) versus large piglets.

Hepatic transglutaminase activity tended to be higher (P=.078) in IUGR piglets (1.56±0.34 mU/g liver) compared to large littermates (1.26±0.13 mU/g liver).

3.4. BHMT and CGL gene expression

The expressions of BHMT and CGL were normalized to β-actin expression in each sample and normalized to a calibrator sample to allow for direct comparison between all piglets, giving expression values in arbitrary units (AU). Betaine-homocysteine methyltransferase expression did not differ between IUGR (1.00±0.24 AU) and large piglets (0.99±0.21 AU) (P=.96). Large piglets had higher CGL expression than IUGR (1.24±0.54 AU versus 0.85±0.54 AU, respectively) (P=.01).

3.5. Global DNA methylation in the liver

Global DNA methylation was estimated by measuring the DPM/0.5 μg DNA in *Hpa*II digests. The DNA methylation in IUGR piglets (3894±469 DPM/0.5 μg DNA) did not differ from that in large piglets (3928±314 DPM/0.5 μg DNA) (P=.88), and birth weight did not correlate with global methylation (P=.623).

4. Discussion

Consistent with epidemiological data, we [16,17] and others [18,19] have demonstrated that the IUGR runt pig develops biomarkers for metabolic syndrome early in adulthood compared to higher-birth-weight littermates. Runts are born to adequately nourished mothers as a result of a decrease in fetal nutrient delivery via reduced blood supply [33]. This porcine model of IUGR allows for the examination of the consequences of a poor intrauterine environment on early postnatal metabolism, while controlling for maternal nutrition. The IUGR neonate's epigenetic profile is likely responsible for the permanent changes in gene expression that translate to higher risk for diseases later in life [14]. Because the

Table 2
Plasma concentrations of amino acids and urea in large and IUGR littermate piglets

	Large	IUGR
Sulfur metabolism amino acids, μM		
Methionine	16.0±3.5	15.6±3.4
Homocysteine	29.6±4.7	27.7±5.0
Cysteine	99.1±14.4*	86.6±4.2
Taurine	140±20	127±24
Serine	373±142	415±87
Glycine	919±182	842±116
Indispensable amino acids, μM		
Arginine	203±44	238±67
Histidine	55.5±25.7	92.3±59.9*,a
Isoleucine	147±39	181±37*,a
Leucine	218±65	260±83
Lysine	261±103	333±132
Phenylalanine	101±12	113±35
Threonine	169±27	203±38
Tryptophan	39.6±6.7	34.7±11.7
Valine	324±47	357±66
Dispensable amino acids, μM		
Alanine	644±103	841±85*
Asparagine	107±24	137±53
Aspartate	38.4±11.8	34.6±6.8
Citrulline	96.9±36.1	90.8±29.1
Glutamate	175±52	246±62*
Glutamine	403±82	468±181
Hydroxyproline	115±15	128±15*
Ornithine	131±89	122±17
Proline	827±176	921±262
Tyrosine	212±38	259±80
Urea, mM	4.79±1.21*	3.42±0.93

Results are mean±S.D. (n=6 per group).
[a] Sex included in the model.
* P<.05.

Table 3
Liver concentrations of free amino acids in large and IUGR littermate piglets

	Large	IUGR
Sulfur metabolism amino acids, nmol/g		
Methionine	326±84	348±107
SAM	68.4±29.0	79.8±31.7
SAH	58.2±20.6	69.2±23.9
SAM/SAH	1.19±0.36	1.19±0.35
Homocysteine	739±240 *, a	532±264
Cystathionine	2.69±0.68	3.63±0.29 *, a
Cysteine	2637±930	2503±430
Taurine	8447±1138 *	6475±900
Serine	2239±292	2228±583
Glycine	5542±836	5366±1167
Indispensable amino acids, nmol/g		
Arginine	78.5±21.1	70.7±24.1
Histidine	36.1±11.4	38.1±21.2
Isoleucine	486±81	535±125
Leucine	1059±236	1213±199
Lysine	571±134	482±191
Phenylalanine	412±90	453±120
Threonine	757±171	747±215
Valine	1446±221	1464±218
Dispensable amino acids, nmol/g		
Alanine	3124±659	3222±581
Aspartate	2628±404	2770±656
Citrulline	348±94	326±143
Glutamate	1959±241	2409±719
Glutamine	455±91	392±164
Hydroxyproline	242±27	243±37
Ornithine	591±228	510±228
Proline	1874±301	1925±368
Tyrosine	502±166	566±156

Results are mean±S.D. (n=6 per group).
[a] Sex included in the model.
* P<.05.

methyl groups required for DNA methylation are generated by the methionine and folate cycles, we hypothesized that, as a result of a poor intrauterine environment (i.e., runts), the activities of the key enzymes in these cycles are altered.

Indeed, this study demonstrated that sulfur amino acid metabolism in IUGR pigs differed from their large littermates. Specific activities of enzymes controlling the disposal of homocysteine, namely, BHMT and CGL, were significantly lower in IUGR piglets. Moreover, the overall capacity of BHMT and CGL per kg of body weight was also significantly lower in IUGR than large piglets, despite the significantly lower body weight in IUGR piglets. Taken as a whole, the double bottleneck of reduced capacity to remethylate and transsulfurate homocysteine in IUGR piglets could potentially limit flux through these pathways. If IUGR neonates have a reduced ability to cope with excess sulfur amino acids, as suggested by this study in IUGR piglets, then protein-rich diets could perturb transmethylation reactions; moreover, diets based on casein, which is rich in methionine (2.70%) and poor in cysteine (0.41%) [34], could be particularly worrisome.

Table 4
Specific activities (nmol product/min/mg protein) of hepatic enzymes of methionine and homocysteine metabolism in large and IUGR littermate piglets

	Large	IUGR	Correlation to body weight (r)
Remethylation			
MS	0.29±0.10	0.29±0.08	0.01
BHMT	0.35±0.06 *	0.25±0.06	0.53 **
MTHFR	0.23±0.02	0.23±0.02	−0.24
Methionine transsulfuration			
MAT	0.37±0.15	0.36±0.06	−0.28
CBS	3.81±0.70	3.68±2.08	−0.01
CGL	5.29±1.53 *	4.23±1.72	0.44 ***

Results are mean±S.D. (n=6 per group).
* P<.05.
** P=.07, linear regression.
*** P=.22, linear regression.

Table 5
Total hepatic capacities (nmol of product/min/kg body weight) of enzymes of large and IUGR littermate piglets, corrected for body weight

	Large	IUGR	Correlation to body weight (r)
Remethylation			
MS	2.02±0.72	1.96±0.58	0.05
BHMT	2.43±0.64 *	1.71±0.46	0.49 **
MTHFR	1.57±0.20	1.59±0.16	−0.07
Methionine transsulfuration			
MAT	2.49±1.01	2.49±0.51	−0.27
CBS	26.7±6.9	25.4±14.8	0.03
CGL	36.6±11.3 *	29.2±13.0	0.43 ***

Results are mean±S.D. (n=6 per group).
* P<.05.
** P=.10, linear regression.
*** P=.15, linear regression.

Betaine-homocysteine methyltransferase is involved in the removal of homocysteine via the remethylation pathway (Fig. 1) [35]. Remethylation allows methionine to be regenerated from homocysteine by using methyl groups from serine via the folate cycle (via MS) or by using methyl groups from betaine (via BHMT). Methionine synthase is considered the main enzyme involved in the remethylation process, whereas BHMT has been considered to be the minor pathway [35]. However, BHMT activity is known to increase to compensate for decreased MS activity as a result of folate deficiency [36]. The converse is also true [36]; even though we did not observe a higher MS capacity in IUGR pigs, it is possible that flux via MS was increased in IUGR piglets to compensate for lower BHMT capacity. Moreover, because decreased choline and betaine intakes are associated with higher risk for neural tube defects, independent of folate intakes [37,38], the role of BHMT in overall methylation status has likely been underestimated.

When remethylation is limited, transsulfuration increases to accommodate the flux from transmethylation; otherwise, homocysteine concentrations escalate [35]. Cystathionine β-synthase and CGL are involved in the catabolism of homocysteine to cysteine in a two-step process (Fig. 1); homocysteine is first condensed with serine by CBS to form cystathionine, and then cystathionine is catabolized to cysteine and α-ketobutyrate by CGL, also known as cystathionase [35]. Cystathionine β-synthase activity did not differ between IUGR and large piglets. However, because CGL activity was significantly lower in IUGR than large piglet livers, the ability of IUGR piglets to clear cystathionine and synthesize cysteine and taurine was reduced. Indeed, IUGR piglets in our study had lower concentrations of plasma total cysteine and hepatic taurine with an apparent accumulation in hepatic cystathionine. It has previously been suggested that CGL is rate limiting for cysteine and taurine synthesis, and its lower activity in neonates (particularly premature neonates) has led to the hypothesis that cysteine may be conditionally essential in early life because methionine cannot be transsulfurated adequately [10,11]. Our data support this clinical observation and suggest that IUGR further limits the activity of this enzyme, thereby limiting the IUGR neonate's capacity to remove cystathionine and synthesize cysteine and taurine. This limited capacity again questions the use of casein as a protein source in neonates; because casein is rich in methionine and poor in cysteine, the conversion of methionine to cysteine is critical to maintaining sulfur amino acid adequacy when casein-based diets are fed. Cysteine could become the first limiting amino acid even in the presence of excess methionine, if this conversion is limited.

Because IUGR piglets had lower capacity to both remethylate and transsulfurate homocysteine, we expected homocysteine concentrations to be elevated. However, homocysteine concentrations were similar in plasma and were actually lower in livers of IUGR piglets. The

lower hepatic accumulation of homocysteine could be due to sufficient MS capacity to deal with any excess homocysteine and/or due to adequate CBS and low CGL activities that led to the hepatic accumulation of cystathionine. Alternatively, IUGR pigs likely have a lower methionine flux as a result of the IUGR pig's lower protein metabolism due to reduced intakes [39]; indeed, a lower protein turnover in IUGR pigs is supported by their lower plasma urea concentrations in this study. Moreover, hyperhomocysteinemia could have been avoided because these piglets were sow-fed, and sow milk contains more appropriate amounts of total protein as well as methionine and cysteine (i.e., methionine to cysteine ratio of 1.40, compared to 0.81 in human milk) [40]. It is possible that, with high-protein feeding or with feeding of casein-based diets (methionine to cysteine ratio of 6.60) [34], the reduced BHMT and CGL capacities may limit remethylation to methionine and eventually lead to hyperhomocysteinemia.

To assess the level of regulation of enzyme activity, gene expression of BHMT and CGL was analyzed in livers by real-time reverse transcriptase-PCR. Cystathionine γ-lyase activity differences between the IUGR and large piglets were due to regulation at the transcriptional level, as CGL expression was significantly lower in the IUGR piglet livers. In contrast, liver BHMT expression was not significantly different between groups; this would suggest that the differences in BHMT activity between IUGR and large piglets may be due to some type of posttranscriptional mechanism. One such possible mechanism for the down-regulation of BHMT activity in IUGR pigs could be related to liver transglutaminases, which have been shown to intra- and intermolecularly cross-link BHMT subunits and thereby reduce BHMT activity *in vitro* [41]. Indeed, IUGR piglets in this study tended to have higher transglutaminase activity ($P=.078$). Posttranscriptional mechanisms of regulation may be more transient than regulation at an expression level and may be related to the availability of substrate. This type of regulation in BHMT activity would be congruent with the very high levels of BHMT found in the liver and could be related to BHMT's role in maintaining remethylation flux in response to MS activity and folate deficiency [35,36].

Because we have recently found that IUGR Yucatan miniature piglets develop early biomarkers for chronic diseases in adulthood [16,17], ultimately, we were interested in whether changes in sulfur amino acid metabolism in IUGR piglets would translate into changes in DNA methylation, possibly explaining the higher risk for developing diseases later in life. Because dietary methyl group donor (folate, vitamin B12, choline and methionine) supplementation can permanently alter epigenetically determined phenotypes [42,43], sulfur amino acid metabolism pathways must be involved in this programming. However, we did not observe any changes in hepatic global DNA methylation or in concentrations of SAM, SAH or its ratio. The SAM/SAH ratio has been called the "methylation index" and is sometimes considered a reflection of methyl group availability [44]. However, it should be noted that global methylation status, which can affect overall chromosomal stability and may contribute to the development of cancer [13], should be differentiated from promoter-specific methylation, which can modify expression of specific genes and is more likely to be the mechanism involved in programming of risk for adult diseases [13,14]. So although we did not observe any changes in global methylation status, it is still possible that promoter-specific changes occurred. Once we identify epigenetically regulated candidate genes, we will use bisulfite sequencing to confirm this.

Reduced prenatal growth in Yucatan miniature piglets impairs their remethylation capacity as well as their ability to remove cystathionine and synthesize cysteine and taurine, which could have important implications on long-term health outcomes. This research has immediate implications in the design of postnatal nutritional therapies for intrauterine growth-retarded infants. For example, the strategy of feeding protein-rich (and especially casein-rich) diets to infants in order to stimulate growth may need to be reevaluated. Furthermore, it is hoped that this research will contribute to the body of knowledge aimed at preventative measures that will help decrease the incidence of the implicated chronic diseases in the fetal origins of adult disease phenomenon.

References

[1] Barker DJ. Fetal origins of coronary heart disease. BMJ 1995;311:171–4.
[2] McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 2005;85:571–633.
[3] Bertram CE, Hanson MA. Animal models and programming of the metabolic syndrome. Brit Med Bull 2001;60:103–21.
[4] Hales CN, Ozanne SE. The dangerous road of catch-up growth. J Physiol 2003;547:5–10.
[5] Rees WD, Hay SM, Buchan V, Antipatis C, Palmer RM. The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply. Br J Nutr 1999;81:243–50.
[6] Rees WD, Hay SM, Brown DS, Antipatis C, Palmer RM. Maternal protein deficiency causes hypermethylation of DNA in the livers of rat fetuses. J Nutr 2000;130:1821–6.
[7] Rees WD. Manipulating the sulfur amino acid content of the early diet and its implications for long-term health. Proc Nutr Soc 2002;61:71–7.
[8] de la Calle M, Usandizaga R, Sancha M, Magdaleno F, Herranz A, Cabrillo E. Homocysteine, folic acid and B-group vitamins in obstetrics and gynaecology. Eur J Obstet Gynecol Reprod Biol 2003;107:125–34.
[9] Osganian SK, Stampfer MJ, Spiegelman D, Rimm E, Cutler JA, Feldman HA, et al. Distribution of and factors associated with serum homocysteine levels in children: Child and Adolescent Trial for Cardiovascular Health. JAMA 1999;281:1189–96.
[10] Zlotkin SH, Anderson GH. The development of cystathionase activity during the first year of life. Pediatr Res 1982;16:65–8.
[11] Viña J, Vento M, García-Sala F, Puertes IR, Gascó E, Sastre J, et al. L-Cysteine and glutathione metabolism are impaired in premature infants due to cystathionase deficiency. Am J ClinNutr 1995;61:1067–9.
[12] Gahl MJ, Benevenga NJ, Crenshaw TD. Rates of lysine catabolism are inversely related to rates of protein synthesis when measured concurrently in adult female rats induced to grow at different rates. J Nutr 1998;128:1503–11.
[13] Kim YI. Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr 2005;135:2703–9.
[14] Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 2004;20:63–8.
[15] Wainfan E, Dizik M, Stender M, Christman JK. Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. Cancer Res 1989;49:4094–7.
[16] Myrie SB, McKnight LL, Van Vliet BN, Bertolo RF. Low birth weight is associated with reduced nephron number and increased blood pressure in adulthood in a novel spontaneous intrauterine growth-restricted model in Yucatan miniature swine. Neonatology 2011;100:380–6.
[17] Myrie SB, McKnight LL, Van Vliet BN, Bertolo RF. Early development of excess visceral fat is associated with the development of metabolic syndrome in small birth weight pigs. Appl Physiol Nutr Metab 2008;33:627.
[18] Poore KR, Fowden AL. The effect of birth weight on hypothalamo-pituitary-adrenal axis function in juvenile and adult pigs. J Physiol 2003;547:107–16.
[19] Poore KR, Fowden AL. The effects of birth weight and postnatal growth patterns on fat depth and plasma leptin concentrations in juvenile and adult pigs. J Physiol 2004;558:295–304.
[20] Puiman P, Stoll B. Animal models to study neonatal nutrition in humans. Curr Opin Clin Nutr Metab Care 2008;11:601–6.
[21] Vester B, Rasmussen K. High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. Eur J Clin Chem Clin Biochem 1991;29:549–54.
[22] Ratnam S, Wijekoon EP, Hall B, Garrow TA, Brosnan ME, Brosnan JT. Effects of diabetes and insulin on betaine-homocysteine *S*-methyltransferase expression in rat liver. Am J Physiol Endocrinol Metab 2006;290:E933–9.
[23] Seo SS. High performance liquid chromatographic determination of homocysteine and cystathionine in biological samples by derivatization with 6-aminoquinolyl-*N*-hydroxylsuccinimidyl carbamate (AQC). J Korean Chem Soc 2005;49:278–82.
[24] Bidlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. J Chromatogr 1984;336:93–104.
[25] Koblin DD, Watson JE, Deady JE, Stokstad EL, Eger 2nd EI. Inactivation of methionine synthetase by nitrous oxide in mice. Anesthesiology 1981;54:318–24.
[26] Garrow TA. Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. J Biol Chem 1996;271:22831–8.
[27] Engbersen AM, Franken DG, Boers GH, Stevens EM, Trijbels FJ, Blom HJ. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. Am J Hum Genet 1995;56:142–50.
[28] Duce AM, Ortiz P, Cabrero C, Mato JM. *S*-adenosyl-L-methionine synthetase and phospholipid methyltransferase are inhibited in human cirrhosis. Hepatology 1988;8:65–8.

[29] Taoka S, Ohja S, Shan X, Kruger WD, Banerjee R. Evidence for heme-mediated redox regulation of human cystathionine beta-synthase activity. J Biol Chem 1998;273:25179–84.
[30] Stipanuk MH. Effect of excess dietary methionine on the catabolism of cysteine in rats. J Nutr 1979;109:2126–39.
[31] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[32] Pogribny I, Yi P, James SJ. A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands. Biochem Biophys Res Commun 1999;262:624–8.
[33] Holt RI, Byrne CD. Intrauterine growth, the vascular system, and the metabolic syndrome. Semin Vasc Med 2002;2:33–43.
[34] National Research Council. Nutrient requirements of swine. 10th ed. Washington (DC): National Acadamies Press; 1998 [chapter 11].
[35] Stipanuk MH. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annu Rev Nutr 2004;24: 539–77.
[36] Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr 2002; 132:2333S–5S.
[37] Shaw GM, Finnell RH, Blom HJ, Carmichael SL, Vollset SE, Yang W, et al. Choline and risk of neural tube defects in a folate-fortified population. Epidemiology 2009;20:714–9.
[38] Shaw GM, Carmichael SL, Yang W, Selvin S, Schaffer DM. Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. Am J Epidemiol 2004;160:102–9.
[39] Ritacco G, Radecki SV, Schoknecht PA. Compensatory growth in runt pigs is not mediated by insulin-like growth factor I. J Animal Sci 1997;75:1237–43.
[40] Davis TA, Nguyen HV, Garcia-Bravo R, Fiorotto ML, Jackson EM, Lewis DS, et al. Amino acid composition of human milk is not unique. J Nutr 1994;124:1126–32.
[41] Ichikawa A, Ohashi Y, Terada S, Natsuka S, Ikura K. In vitro modification of betaine-homocysteine *S*-methyltransferase by tissue-type transglutaminase. Int J Biochem Cell Biol 2004;36:1981–92.
[42] Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 2003;23:5293–300.
[43] Waterland RA, Dolinoy DC, Lin JR, Smith CA, Shi X, Tahiliani KG. Maternal methyl supplements increase offspring DNA methylation at Axin Fused. Genesis 2006;44: 401–6.
[44] Van den Veyver IB. Genetic effects of methylation diets. Annu Rev Nutr 2002;22: 255–82.