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ORIGINAL ARTICLE

Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep

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Abstract Intrauterine growth restriction is a significant problem worldwide, resulting in increased rates of neonatal morbidity and mortality, as well as increased risks for metabolic and cardiovascular disease. The present study investigated the role of maternal undernutrition and L-arginine administration on fetal growth and development. Embryo transfer was utilized to generate genetically similar singleton pregnancies. On Day 35 of gestation, ewes were assigned to receive either 50 or 100% of their nutritional requirements. Ewes received i.v. injections of either saline or L-arginine three times daily from Day 100 to Day 125. Fetal growth was assessed at necropsy on Day 125. Maternal dietary manipulation altered circulating concentrations of leptin, progesterone, and amino acids in maternal plasma. Fetal weight was reduced in nutrient-restricted ewes on Day 125 compared with 100% fed ewes. Compared with saline-treated underfed ewes, maternal L-arginine administration did not affect fetal weight but increased weight of the fetal pancreas by 32% and fetal peri-renal brown adipose tissue mass by 48%. These results indicate that L-arginine administration enhanced fetal pancreatic and brown adipose tissue development. The postnatal effects of increased pancreatic and brown adipose tissue growth warrant further study.

Keywords Adipose tissue · Pregnancy · Fetus · Arginine · Sheep · Nutrient restricted

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Abbreviations

BAT	Brown adipose tissue
EIA	Enzyme-linked immunoassay
FUV	Fetal umbilical vein
IUGR	Intrauterine growth restriction
NO	Nitric oxide
NRC	National Research Council
WAT	White adipose tissue

Introduction

Maternal nutrition is a primary determinant of fetal growth. Current estimates suggest that intrauterine fetal growth restriction (IUGR) occurs in 7–15% of pregnancies (Alexander et al. 2003; Baschat 2004). Insufficient availability and/or delivery of nutrients to the conceptus alter placental growth and function, resulting in IUGR in a number of species including rats, sheep, pigs, and humans (Wu et al. 2004). In humans, IUGR can arise from several causes, including (1) placental insufficiency, which is characterized by fetal hypoxia and damage to the placental vascular bed and (2) a number of nutritionally related problems including multi-fetal pregnancy, short duration between pregnancies, hyperemesis gravidarium, or maternal malnutrition, which remains a common concern in developing countries (McMillan and Robinson 2005). Fetal growth restriction has been implicated as a contributing factor to a number of metabolic disorders and adult onset diseases, such as type-II diabetes, hypertension, impaired glucose tolerance, and obesity (Wu et al. 2006). There is increasing evidence that nutrient availability to the developing fetus programs their metabolic state such that

growth-restricted fetuses have a "thrifty" metabolic phenotype, resulting in obesity during postnatal life when available nutrients exceed those necessary to meet metabolic demands (Hales and Barker 2001).

During pregnancy, transport of nutrients to the fetus(es) is dependent on uterine blood flow which increases considerably during the second and third trimesters of gestation (Thaler et al. 1990; Lang et al. 2003). Indeed, indices of uterine blood flow are reduced in pregnancies complicated by IUGR (Bower et al. 1998; Moore et al. 2008). Nitric oxide (NO), a product of arginine catabolism, plays a crucial role in regulating placental angiogenesis (the growth of new vessels from the existing vasculature) and fetal-placental blood flow during gestation (Sladek et al. 1997; Reynolds and Redmer 2001; Bird et al. 2003). In addition to NO, polyamines are essential to placental growth and angiogenesis and, therefore, for increasing uterine and placental–fetal blood flow (Wu et al. 2006). Of particular note, arginine metabolism results in the production of both NO by NO synthase and polyamines via ornithine formation, and ornithine decarboxylase in placentae (Wu et al. 2004; 2008). Feeding arginine-free diets to pregnant rats or inhibiting NO synthesis results in increased fetal resorptions, IUGR, increased perinatal mortality, and a decrease in the number of live fetuses at birth (Greenberg et al. 1997). Thus, endogenous synthesis of arginine is insufficient for pregnant dams and must be provided from diets to support fetal survival and growth (Wu et al. 2009). Interestingly, maternal arginine supplementation reduces the incidence of fetal growth restriction in sheep and humans and increases the live-born litter weight in rats and pigs (Vosatka et al. 1998; Xiao and Li 2005; Mateo et al. 2007; Zeng et al. 2008; Lassala et al. 2010). These findings provide a strong experimental basis for the use of arginine to prevent and treat IUGR in animals and humans.

Fetal programming of the metabolic state and the resultant development of adipose tissue have drawn considerable interest in an effort to ameliorate global obesity (Wang et al. 2011). Specifically, accumulation of additional brown adipose tissue (BAT) as opposed to white adipose tissue (WAT) is hypothesized to be beneficial to improving the metabolic state, as BAT is highly efficient at burning fatty acids and glucose via a unique uncoupling protein and releasing it as heat (Cannon and Nedergaard 2004). In the fetal lamb, BAT development increases rapidly between Days 70 and 120 of gestation and then slows to term (Day 147) (Alexander 1978). The primary site of BAT accretion in the lamb occurs in the peri-renal region, with secondary sites near the heart and the vertebrae. Although fetal WAT is known to be markedly affected by prolonged manipulation of maternal feed intake (Gopalakrishnan et al. 2001; Bispham et al. 2003), the effect of maternal nutrition on fetal BAT development and function is largely unknown (Satterfield and Wu 2011). There is compelling evidence that physiological levels of NO enhance mitochondrial biogenesis and function in non-BAT (Nisoli et al. 2004a, b; Clementi and Nisoli 2005). However, little is known about a role for NO in regulating fetal BAT growth and metabolism in any species. Interestingly, brown adipocytes contain a disproportionately high number of mitochondria compared with cells of other tissues (Sell et al. 2004). In addition, development of the vast network of capillaries that supply nutrients and oxygen to brown adipocytes and redirect heat produced by BAT may also be under NO control (McKnight et al. 2010).

This study tested the hypothesis that maternal arginine treatment increases fetal growth by increasing nutrient delivery to the fetus. The objectives were to assess the effect of maternal arginine supplementation on maternal metabolism as well as fetal growth and development in underfed pregnant ewes.

Materials and methods

All experimental and surgical procedures were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

Experimental design

Prior to and after embryo transfer, recipient ewes were fed 100% of their National Research Council (NRC 1985) requirements to maintain their body condition. Contents of the diet can be found in Table 1. Ewes were synchronized into estrus and a single embryo from a superovulated Suffolk ewe of normal body condition was transferred into the uterus on Day 5.5 post-estrus. Pregnancy was diagnosed by ultrasound on Day 28 of gestation. On Day 35 of pregnancy, ewes were randomly assigned to a control-fed group (100% NRC) ($n = 10$) and a nutrient-restricted group (50% NRC requirements) ($n = 24$). All ewes were individually housed from Day 28 to Day 125 of gestation. Beginning on Day 28 of gestation, body weight was analyzed every 7 days and feed intake was adjusted based on changes in body weight. Both nutrient-restricted and control-fed ewes consumed their entire dietary allotment each day. On Day 100, nutrient-restricted ewes were further assigned randomly to receive either sterile saline ($n = 12$) as a control or L-arginine-HCl ($n = 12$) (equivalent to 81 mg L-arginine/kg bodyweight/day) dissolved in saline (pH 7.2) and filter-sterilized (Lassala et al. 2010). All 100% NRC ewes were administered sterile saline as normal-fed controls. Treatments were administered via i.v. infusion at 06:00, 14:00, and 22:00 h daily beginning on Day 100 until

Table 1 Composition of the diet for pregnant sheep[a]

Ingredients	Content (%; as-fed basis)
Soybean hulls	31.05
Wheat midds	27.3
Corn	19.74
Soybean meal	5.5
Rice bran	5.0
Dehydrated Alfalfa (17%)	5.0
Soy oil	2.5
Liquid binder	2.5
Sodium bicarbonate	0.50
Salt	0.50
Ground limestone	0.30
Vitamin mixture[b]	0.06
Mineral mixture[c]	0.05

[a] Provided the following (% of diet): dry matter, 90.2; crude protein, 12.8; crude fat, 5.36; crude fiber, 15.9; calcium, 0.51; phosphorus, 0.48; sodium, 0.40; potassium, 1.08; sulfur, 0.18; magnesium, 0.25. The content of amino acids in the diet (%; as-fed basis) is as follows: alanine, 0.84; arginine, 0.76; asparagine, 0.66; aspartate, 0.75; cysteine, 0.24; glutamate, 1.01; glutamine 1.16; glycine, 0.63; histidine, 0.28; isoleucine, 0.54; leucine, 1.07; lysine, 0.64; methionine, 0.21; phenylalanine, 0.63; proline, 1.02; serine, 0.65; threonine, 0.49; tryptophan, 0.16; tyrosine, 0.48; and valine, 0.64

[b] Provided the following (mg/kg of the complete diet): retinyl acetate, 10.9; D-α-tocopherol acetate, 22.0; thiamin, 3.0; menadione sodium bisulfate, 1.3; cholecalciferol, 0.041

[c] Provided the following (mg/kg of the complete diet; ppm): manganese, 168; iron, 134; copper, 14.4; cobalt, 0.29; zinc, 152; iodine, 1.00; selenium, 0.29; and molybdenum, 1.00

Day 125 of gestation. Blood samples from the maternal jugular vein were collected into heparinized tubes on Days 113 and 125 of gestation. Plasma was obtained following centrifugation (2,000×*g* for 10 min at 4°C) and stored at −20°C until analysis. On Day 125 of pregnancy, when ovine fetal growth is in the middle of the exponential growth phase (Kwon et al. 2003), conceptus (fetal-placental unit) development was determined at necropsy.

Tissue collection and handling following necropsy

At the time of necropsy, ewes were stunned using a captive bolt gun followed immediately by exsanguination. Immediately upon exsanguination, the uterus was exposed and venous blood samples were collected from the uterine vein into a heparinized tube. Plasma was harvested following centrifugation (2,000×*g* for 10 min at 4°C) and then stored at −20°C until analysis. Amniotic and allantoic fluids were obtained and volumes recorded by puncturing the amniochorion and chorioallantois, respectively (Kwon et al. 2003). Following collection of the fetal fluids and umbilical artery and umbilical vein blood samples, the fetus was removed, weighed, measured, and dissected. Samples from fetal organs were preserved in either 4% paraformaldehyde or snap frozen in liquid nitrogen and stored at −80°C for analyses. Following dissection of the fetus, the fetal carcass was further broken down into soft tissue and bone by scalpel dissection. Fetal bone was weighed and discarded while fetal soft tissues were frozen at −20°C for later analyses. The placenta was further dissected to isolate all placentomes for the assessment of placentome number, gross morphology, and weight. Placentomes were fixed in either 4% paraformaldehyde or snap frozen in liquid nitrogen. Simultaneously, the maternal organs were dissected and weighed. Upon removal of maternal organs, the pelt and head were removed and the carcass was split along the spine. One half of the remaining carcass was broken down by knife to collect all soft tissues and bone for subsequent carcass composition analyses. Bone was weighed immediately and discarded while soft tissues were frozen at −20°C for analyses.

Analyses of carcass ash, dry matter, and total lipid content

For analyses of maternal carcass composition, soft tissues were thawed and subsequently homogenized using a Seydelmann Cutter K64 (Strasser; Stuttgart, Germany). A subsample of the homogenized soft tissue was further homogenized in a food processor, aliquoted, and stored at −20°C. Fetal soft tissues were homogenized in a food processor, aliquoted, and stored at −20°C. Total lipids from soft tissue were extracted from homogenates of ~1 g samples using a mixture of chloroform and methanol (2:1, vol/vol) (Folch et al. 1957). Total nitrogen in tissues were analyzed in approximately 1.0 g samples using LECO Model FP-528 Analyzer (St. Joseph, MI, USA) (Li et al. 2011), and crude protein was calculated as total nitrogen multiplied by 6.25 on the basis of the assumption that protein contains 16% N (Wu et al. 1997). Ash was determined by placing tissues in a 550°C furnace for 12 h.

Analyses of amino acids and glucose in maternal and fetal plasma

Fetal and maternal plasma (0.5 ml, Day 125) were deproteinized with an equal volume of 1.5 M $HClO_4$, followed by addition of 0.25 ml 2 M K_2CO_3 (Wu et al. 1996). Amino acids in the extract were determined by fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde as described previously (Wu et al. 1997). The integration of chromatographic peaks was performed using Millenium-32 Software (Waters, Milford, MA, USA). Glucose was determined enzymatically from maternal plasma collected on Day 113 using a

spectrophotometric method involving hexokinase and glucose-6-phosphate dehydrogenase as previously described (Fu et al. 2005).

Hormone analyses

Concentrations of leptin in maternal plasma on Day 113 were determined by specific RIA as previously described (Delavaud et al. 2000). Concentrations of progesterone in plasma on Day 113 were determined according to manufacturer's specifications using an antiserum highly specific for progesterone (DSL-3900 ACTIVE Progesterone Coated-Tube Radioimmunoassay Kit, Diagnostic Systems Laboratories, Webster, TX, USA) as previously described (Satterfield et al. 2006). Concentrations of insulin in maternal plasma on Day 113 were assayed using an ovine-specific EIA (80-INSOV-E01, ALPCO Diagnostics, Salem, NH, USA) according to manufacturer's instructions. Assay results were calculated using the AssayZap Version 3.1 program (Biosoft, Ferguson, CA, USA). Intrassay coefficients of variation were less than 7% for all hormone assays.

Statistical analyses

A total of six ewes were removed from the study (four in the 100% NRC-fed group, one in the 50% NRC-fed group, and one in the 50% NRC-fed arginine-treated group) due to death (3 ewes), inappropriately timed fetal collection (1 ewe), extremely unhealthy and/or dying fetus (1 ewe), and an unexpected twin pregnancy (1 ewe). Statistical analyses of maternal weight changes were determined by two-way ANOVA for repeated measures using Day and treatment as independent variables and the presence of an interaction between Day and treatment (Wei et al. 2011). All other maternal and fetal measures were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC, USA) with preplanned pair-wise comparisons. Fetal organ weights presented are relative weights after adjustment for fetal weight using fetal weight as a covariate in the statistical model. Data are presented as the least-squares means (LSM) with standard error of the mean (SEM). Differences in means were considered to be statistically significant when a P value was ≤ 0.05 while a P value of <0.1 was considered to indicate a tendency toward significance.

Results

Maternal bodyweight changes

As expected, dietary treatment altered ($P < 0.01$) maternal bodyweight (Table 2). The initiation of nutrient restriction on Day 35 decreased ($P < 0.01$) maternal bodyweight in 50% fed as compared with 100% fed ewes by Day 63 and maternal bodyweight remained lower till Day 125. Maternal arginine administration from Day 100 to 125 did not alter ($P > 0.10$) maternal bodyweight within nutrient-restricted ewes.

Maternal endocrine status

As expected, maternal nutrient restriction resulted in reduced ($P < 0.01$) concentrations of leptin on Day 113 of gestation as compared with 100% NRC-fed ewes (Table 3). Concentrations of progesterone were elevated ($P < 0.01$) in nutrient-restricted ewes in comparison with 100% NRC-fed controls. Maternal arginine supplementation did not affect concentrations of leptin or progesterone within nutrient-restricted ewes. Concentrations of insulin and glucose did not differ ($P > 0.10$) between treatments.

Maternal organ, tissue, and placental measures

Maternal nutritional regimen altered weights of a number of maternal organs (Table 4) and influenced measures related to maternal body composition (Table 5). Maternal heart weight and weights of the left and right ventricles of the heart were reduced ($P < 0.05$) in arginine-treated underfed ewes compared with 100% NRC-fed controls. Weights of the maternal liver and stomach ($P < 0.05$) were reduced in both arginine and saline-treated underfed ewes compared with ewes receiving 100% NRC. Weight of the large intestine tended to be reduced ($P = 0.06$) in arginine-treated underfed ewes as compared with saline-treated underfed and control-fed ewes. Amniotic and allantoic fluid volumes, mean and total placentome weights, and placentome number were not affected ($P > 0.10$) by dietary treatment or amino acid supplementation. There was no difference ($P > 0.05$) in the proportion of placentome morphological type (A. B, C, or D) between treatments.

Weight of the maternal gastrocnemius muscle as well as weight of the hindlimb was reduced ($P < 0.01$) in nutrient-restricted ewes as compared with 100% NRC-fed controls (Table 5). Last rib backfat was reduced ($P < 0.05$) in arginine-treated underfed ewes versus control-fed ewes. Weight of internal fat tended to be reduced ($P = 0.07$) in nutrient-restricted ewes versus 100% NRC-fed controls. Maternal carcass soft tissue weight was lower ($P < 0.05$) in nutrient-restricted versus control-fed ewes. Soft tissue dry matter and lipid content were lower ($P < 0.01$) in nutrient-restricted ewes as compared with adequately fed controls. In contrast, soft tissue protein content was greater ($P < 0.01$) in nutrient-restricted versus normal-fed control ewes.

Table 2 Effects of nutrient restriction and arginine administration on maternal body weight

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100% NRC Saline ($n = 6$)	SEM	P value
Day 35 of pregnancy	61.1	64.5	63.4	5.9	>0.10
Day 125 of pregnancy	54.4[a]	57.9[a]	74.8[b]	6.3	<0.001
Weight change (d125–d35)	−6.7[a]	−6.6[a]	11.4[b]	1.4	<0.001

Data are mean values with pooled standard error of the mean (SEM). Within each row, values with different alphabets differ

Table 3 Effects of nutrient restriction and arginine administration on maternal circulating levels of glucose and hormones

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100% NRC Saline ($n = 6$)	SEM	P value
Glucose (mM)	2.68	2.44	2.90	0.23	>0.10
Insulin (ng/ml)	0.5	0.37	0.57	0.1	>0.10
Leptin (ng/ml)	1.9[a]	2.2[a]	3.4[b]	0.17	<0.001
Progesterone (ng/ml)	17.3[b]	15.2[b]	9.7[a]	1.5	<0.01

Data are mean values with pooled standard error of the mean (SEM). Within each row, values with different alphabets differ

Table 4 Effects of nutrient restriction and arginine administration on maternal organ weights and placental measures

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100% NRC Saline ($n = 6$)	SEM	P value
Heart (g)	233[a]	261[a, b]	306[b]	16.3	<0.05
Left ventricle (g)	94[a]	109[a, b]	129[b]	7	<0.05
Right ventricle (g)	59[a]	72[a, b]	85[b]	6	<0.05
Septum (g)	39	39	44	2.9	>0.10
Kidney (g)	130	128	138	9.9	<0.10
Liver (g)	599[a]	556[a]	737[b]	39.3	<0.05
Lung (g)	669	661	685	47.2	>0.10
Adrenal (g)	8	7.6	6.3	1.3	>0.10
Spleen (g)	86	83	109	8.5	>0.10
Pancreas (g)	70	69	79	8.7	>0.10
Small intestine (g)	1261	1093	1354	157.4	>0.10
Large intestine (g)	239[a]	391[b]	384[b]	53.9	0.06
Stomach (kg)	1.6[a]	1.7[a]	2.8[b]	0.26	<0.05
Mammary gland (kg)	0.9	0.8	1.2	0.13	>0.10
Mean placentome weight (g)	7.6	6.9	7.5	0.6	>0.10
Placentome number	67	73	73	3	>0.10
Amniotic fluid (mL)	861	630	907	145	>0.10
Allantoic fluid (mL)	559	529	587	142	>0.10

Data are mean values with pooled standard error of the mean (SEM). Within each row, values with different alphabets differ

Fetal weight, organ weights, and carcass composition

Maternal nutrient restriction decreased ($P < 0.05$) fetal weight at necropsy (Table 6). Maternal arginine treatment had no effect on fetal weight compared with saline-treated underfed ewes. Weight of the fetal pancreas was 32% heavier ($P = 0.05$) in fetuses from arginine-treated versus saline-treated underfed ewes. Weight of the fetal stomach was lower ($P < 0.01$) in fetuses from saline-treated underfed ewes as compared with fetuses from arginine-treated underfed and control-fed ewes. Weight of fetal perirenal brown adipose tissue was increased ($P < 0.01$) in fetuses from 100% NRC-fed and arginine-treated underfed ewes as compared with fetuses from saline-treated underfed ewes. Dry matter content in fetal carcass soft tissue was reduced ($P < 0.01$) in fetuses from arginine-treated underfed ewes as compared with fetuses from both adequately fed and underfed saline-treated ewes.

Table 5 Effects of nutrient restriction and arginine administration on maternal carcass composition

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100% NRC Saline ($n = 6$)	SEM	P value
Gastrocnemius muscle (g)	358[a]	391[a]	560[b]	28.1	<0.001
Whole hindlimb (kg)	1.7[a]	1.7[a]	2.6[b]	0.13	<0.001
Last rib backfat (cm)	0.07[a]	0.2[a, b]	0.41[b]	0.07	<0.05
Bodywall thickness (cm)	0.93	1.04	1.4	0.14	>0.10
Internal fat (g)	294[a]	359[a]	1192[b]	255	0.07
Carcass soft tissue (kg)	8.5[a]	8.4[a]	12.5[b]	0.9	0.05
Carcass bone (kg)	3.8	4.0	3.9	0.3	>0.10
Soft tissue DM content (%)	29.0[a]	27.3[a]	39.8[b]	2	<0.001
Soft tissue lipid content (%)	18.7[a]	17.1[a]	32.1[b]	3.4	<0.01
Soft tissue protein content (%)	70.2[a]	68.9[a]	57.1[b]	3.3	<0.01
Soft tissue ash content (%)	0.98	0.92	0.90	0.05	>0.10

Data are mean values with pooled standard error of the mean (SEM). Within each row, values with different alphabets differ

DM dry matter

Table 6 Effects of nutrient restriction and arginine administration on fetal organ weights

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100% NRC Saline ($n = 6$)	SEM	P value
Fetal weight (kg)	3.5[a]	3.45[a]	3.99[b]	0.13	<0.05
Pancreas (g)	2.9[b]	2.2[a]	2.4[a, b]	0.38	0.05
Stomach (g)	26.6[b]	23.1[a]	28.6[b]	0.57	<0.01
Brown adipose tissue (g)	9.1[b]	6.2[a]	7.2[b]	0.8	<0.001
Soft tissue DM content (%)	18.2[a]	19.9[b]	19.9[b]	0.4	<0.01

Data are mean values with pooled standard error of the mean (SEM). Within each row, values with different alphabets differ

DM dry matter

Maternal and fetal plasma amino acids

Maternal diet and/or arginine administration affected concentrations of a number of amino acids in plasma from maternal jugular vein blood (Table 7). Concentrations of alanine, beta-alanine, citrulline, and tryptophan were reduced ($P < 0.05$) in 50% NRC-fed ewes versus ewes receiving 100% NRC. In contrast, concentrations of lysine in plasma were elevated ($P < 0.01$) in nutrient-restricted versus adequately fed ewes. Concentrations of glutamate tended to be reduced ($P = 0.07$) in saline-treated nutrient-restricted ewes as compared with control-fed ewes. Concentrations of histidine were lower ($P < 0.05$) in arginine-treated underfed ewes as compared with control-fed ewes. Concentrations of arginine and ornithine were greater ($P < 0.01$) in arginine-treated underfed ewes as compared with saline-treated ewes of either dietary treatment.

Concentrations of amino acids in fetal umbilical vein (FUV) plasma were affected by maternal diet and/or maternal arginine administration (Table 8). Concentrations of glutamate and taurine in FUV plasma were reduced ($P < 0.05$) nutrient-restricted versus control-fed ewes. Concentrations of beta-alanine ($P < 0.05$) and serine ($P = 0.078$) were reduced in FUV plasma of arginine-treated versus control-fed ewes. In contrast, arginine treatment increased ($P < 0.01$) concentrations of ornithine in FUV plasma as compared with saline-treated ewes regardless of level of nutritional intake.

Discussion

We recently reported that parenteral L-arginine administration from Day 60 of gestation to term increased birth weights of lambs from nutrient-restricted ewes (Lassala et al. 2010). In the present study, we evaluated the effects of maternal arginine supplementation on maternal, fetal, and placental parameters in nutrient-restricted ewes. Results of the present study indicate that a 25-day period of arginine administration during late gestation is insufficient to increase fetal weight on day 125. Nonetheless, maternal arginine administration increased peri-renal BAT accretion by 48% in fetuses from nutrient-restricted ewes. This striking observation has significant potential impact for improving neonatal thermogenesis and survival. In addition, arginine administration increased weight of the fetal pancreas, which may alter insulin production in adulthood.

The incidence of fetal growth restriction in response to maternal undernutrition is a socially and economically important issue for both human medicine and livestock

Table 7 Effects of nutrient restriction and arginine administration on concentrations of amino acids in maternal plasma

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100% NRC Saline ($n = 6$)	SEM	P value
Ala	113[a]	118[a]	179[b]	16	<0.05
Arg	316[b]	149[a]	193[a]	25	<0.001
Asn	26	27	30	2	>0.10
Asp	5	4	6	1	>0.10
β-Ala	14[a]	13[a]	21[b]	2	<0.05
Citrulline	190[a]	181[a]	259[b]	19	<0.05
Gln	211	222	242	13	>0.10
Glu	53[a, b]	47[a]	68[b]	6	0.07
Gly	659	574	768	78	>0.10
His	31[a]	37[a, b]	45[b]	3	<0.05
Ile	79	87	75	6	>0.10
Leu	106	108	86	9	>0.10
Lys	154[b]	143[b]	86[a]	15	<0.01
Met	21	22	27	3	>0.10
Ornithine	247[b]	52[a]	65[a]	17	<0.001
Phe	86	84	86	4	>0.10
Ser	61	65	75	9	>0.10
Taurine	130	115	109	13	>0.10
Thr	48	43	61	8	>0.10
Trp	26[a]	21[a]	39[b]	4	<0.05
Tyr	65	39	78	13	>0.10
Val	126	117	125	14	>0.10

Data, which are expressed as nmol/mL, are means with pooled standard error of the mean (SEM). For each amino acid, values with different alphabets differ

production (Wu et al. 2004, 2006). As fetal growth is limited by the availability of nutrients and placental blood flow is the primary mechanism by which nutrients are delivered to the fetus, therapies to enhance uteroplacental blood flow are hypothesized to be a desirable strategy to ameliorate IUGR in mammals (Reynolds et al. 2006; Wu et al. 2010). Using the undernourished sheep as a model system, we reported that administration of sildenafil citrate (Viagra) to both underfed and adequately fed mothers increased fetal weight of singleton pregnancies by 14% on Day 115 of gestation (Satterfield et al. 2010). Sildenafil citrate sustains levels of cGMP by inhibiting phosphodiesterase 5, an enzyme that hydrolizes cyclic guanosine monophosphate (Glossmann et al. 1999). As arginine is the biological precursor for NO synthesis (Blachier et al. 2011) and can regulate gene expression (Geng et al. 2011; Wu 2010; Wu et al. 2011), its use as a dietary supplement has provided insight into the importance of this amino acid in fetal/placental development. Indeed, feeding arginine-free diets to pregnant rats or inhibiting NO synthesis increases fetal resorptions, IUGR, and perinatal mortality, while decreasing the number of live fetuses born (Greenberg et al. 1997). Using the undernourished sheep model, we found that parenteral L-arginine administration increased birth weight of lambs whose mothers had been nutrient-restricted (Lassala et al. 2010). The latter study was preceded by the observation that direct infusion of arginine into the fetal femoral vein for 3–4 h increased fetal whole-body protein accretion in an ovine model of IUGR induced by placental insufficiency (de Boo et al. 2005). Further, arginine administration for 1 week during late gestation increased systematic NO availability (indicated by nitrite and nitrate concentrations in serum) and birth weight by 6.4% in women (Xiao and Li 2005). In contrast, results of the present study indicate that maternal arginine administration to undernourished ewes between Days 100 and 125 of gestation failed to increase fetal weight above those receiving saline. The apparent contradictory nature of these data in comparison to our previously published work likely results from a shortened duration of arginine supplementation in the present study (25 vs. 87 days). Alternatively, a critical window of supplementation may exist to allow for increased fetal growth. In addition, the present study was limited to singleton-bearing ewes while the previous study involved singletons and twins. It is possible that the additional nutritional drain of carrying an additional fetus could impact the ability of arginine to increase nutrient delivery from the mother to the fetus(es). Supporting evidence for this possibility is the observation that birth weight of lambs from 100% NRC-fed ewes from our previous study (Lassala et al. 2010) is identical to the weights of the Day 125 singleton fetuses collected in the present study (3.99 vs. 4.0 kg, respectively).

Table 8 Effects of nutrient restriction and arginine administration on circulating levels of amino acids in fetal umbilical vein

Diet Treatment	50% NRC Arginine ($n = 11$)	50% NRC Saline ($n = 11$)	100%NRC Saline ($n = 6$)	SEM	*P* value
Ala	303	334	362	25	>0.10
Arg	222	164	197	24	>0.10
Asn	51	63	60	5	>0.10
Asp	12	14	16	1	>0.10
β-Ala	99[a]	160[b]	172[b]	19	<0.05
Citrulline	220	202	215	18	>0.10
Gln	534	521	453	64	>0.10
Glu	89[a]	103[a]	181[b]	24	<0.05
Gly	880	822	607	104	>0.10
His	48	52	56	6	>0.10
Ile	104	102	91	5	>0.10
Leu	182	182	158	11	>0.10
Lys	125	150	123	17	>0.10
Met	27	32	35	4	>0.10
Ornithine	169[b]	108[a]	110[a]	12	<0.001
Phe	135	137	124	6	>0.10
Ser	503[a]	714[b]	758[b]	94	0.08
Taurine	124[a]	185[a]	264[b]	24	<0.001
Thr	189	212	275	29	>0.10
Trp	54	56	59	4	>0.10
Tyr	93	97	86	7	>0.10
Val	279	244	248	25	>0.10

Data, which are expressed as nmol/mL, are mean values with pooled standard error of the mean (SEM). For each amino acid, values with different alphabets differ

Available evidence shows that maternal undernutrition had a significant effect on concentrations of the majority of amino acids in maternal and fetal plasma (Lassala et al. 2010; Satterfield et al. 2010). We have previously reported that administration of sildenafil citrate (which increases cGMP availability and mimics the action of NO in cells) to nutrient-restricted and adequately fed ewes increases concentrations of amino acids in fetal plasma, as well as amniotic and allantoic fluids (Satterfield et al. 2010). The increased concentrations of amino acids likely contributed to the increase in fetal weight observed in the previous study involving sildenafil citrate. In the present work, arginine treatment only resulted in increased concentrations of ornithine [an immediate precursor of polyamines (Wu 2009)] in fetal umbilical vein plasma while decreasing concentrations of serine and beta-alanine. In contrast, administration of sildenafil citrate from Day 28 to 115 of gestation resulted in increased concentrations of 21 out of 25 amino acids in fetal umbilical vein plasma. Functional changes in the placenta of ewes receiving arginine administration for a prolonged period of time may be responsible for enhanced transport of nutrients from the mother to the fetus during late gestation when absolute fetal growth (g/day) is most rapid (Kwon et al. 2003).

A growing body of epidemiological evidence indicates that low birth weight is associated with glucose intolerance and increased risk of developing non-insulin dependent diabetes mellitus in adulthood (Satterfield et al. 2011). It has been postulated that nutrient deficits during development alters the formation of the endocrine pancreas, resulting in abnormal insulin secretion (Avril et al. 2002). In an experimental sheep model of placental insufficiency, pancreatic weight and β-cell percent are reduced in IUGR fetuses in association with reduced pancreatic insulin content (Green et al. 2010). In our model of nutritionally induced IUGR, a reduction in pancreatic weight or placental weight between 50% NRC-fed and 100% NRC-fed fetuses was not observed. However, arginine treatment increased pancreatic growth in fetuses from nutrient-restricted ewes. Other nutritionally based models of IUGR in sheep indicate that low birth weight offspring have an altered rate of postnatal growth and reduced efficiency of feed/forage utilization (Greenwood and Bell 2003; Wu et al. 2006). Future studies are warranted to determine if impaired pancreatic function contributes to the reduced growth and reduced efficiency of nutrient utilization in IUGR offspring and if maternal arginine treatment to enhance pancreatic development can ameliorate these adverse effects of IUGR.

The observation that maternal arginine treatment increased fetal peri-renal BAT has potentially significant

implications to both animal agriculture and medicine. Neonatal survival in mammals, including sheep, depends on the offspring's ability to maintain its core body temperature during periods of cold stress (Symonds and Lomax 1992). In neonates of most species, including humans and sheep, the production of heat to maintain core body temperature is mediated in large part by non-shivering thermogenesis. During this process, a large amount of heat is generated via a unique mitochondrial protein (uncoupling protein 1) that uncouples ATP synthesis from the oxidative process with heat being the primary product (Asakura 2004). In humans, pre-term and low birth weight infants are prone to hypothermia, increasing the incidence of fetal morbidity and mortality (Buetow and Klein 1964; Day et al. 1964; Knobel et al. 2005). In sheep, approximately 40% of total non-predator related lamb deaths are attributed to cold weather-related losses in the U.S. (Simpson 1995). Cold and cold-related starvation is the leading cause of perinatal lamb losses (Samson and Slee 1981). Metabolism of BAT for non-shivering thermogenesis is responsible for approximately 50% of the heat generated in newborn lambs although BAT constitutes only 2% of their body weight at birth (Symonds and Lomax 1992). To date, nutritional strategies have had limited success in increasing ovine fetal BAT deposition in utero, perinatal thermogenesis, or survival of newborn lambs (Lammoglia et al. 1999a, b; Budge et al. 2000; Dietz et al. 2003; Encinias et al. 2004; Chen et al. 2007). We recently reported that arginine treatment increases BAT growth while decreasing white adipose tissue accumulation in adult genetically obese rats (Zucker diabetic fatty rats) and adult diet-induced obese rats (Wu et al. 2007; Jobgen et al. 2009). However, to our knowledge, this is the first report that arginine increases BAT development in the fetus. Although the present study does not elucidate the mechanisms responsible for the effect of arginine on enhancing fetal BAT growth, our work provides the potentially groundbreaking findings for future investigation. Given the major role for BAT in neonatal thermogenesis, the results reported herein have important implications for both biomedicine and livestock production.

In conclusion, parenteral administration of arginine to underfed ewes from Day 100 to 125 of gestation failed to increase fetal weight on Day 125 in singleton pregnancies but increased peri-renal BAT mass. These novel findings, coupled with existing data, provide an experimental basis for the clinical use of arginine for long-term administration to both reduce the incidence of IUGR and enhance neonatal thermogenesis at birth. Improved neonatal thermogenesis in pre-term and growth-restricted infants may reduce the incidence of neonatal morbidity and mortality. Future studies are needed to determine if increased BAT mass following maternal arginine supplementation will result in increased thermogenesis after birth.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Alexander G (1978) Quantitative development of adipose tissue in foetal sheep. Aust J Biol Sci 31:489–503

Alexander GR, Kogan M, Bader D et al (2003) US birth weight/gestational age-specific neonatal mortality: 1995–1997 rates for whites, hispanics, and blacks. Pediatrics 111:e61–e66

Asakura H (2004) Fetal and neonatal thermoregulation. J Nippon Med Sch 71:360–370

Avril I, Blondeau B, Duchene B et al (2002) Decreased beta-cell proliferation impairs the adaptation to pregnancy in rats malnourished during perinatal life. J Endocrinol 174:215–223

Baschat AA (2004) Fetal responses to placental insufficiency: an update. BJOG 111:1031–1041

Bird IM, Zhang L, Magness RR (2003) Possible mechanisms underlying pregnancy-induced changes in uterine artery endothelial function. Am J Physiol Regul Integr Comp Physiol 284:R245–R258

Bispham J, Gopalakrishnan GS, Dandrea J et al (2003) Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. Endocrinology 144:3575–3585

Blachier F, Davila AM, Benamouzig R et al (2011) Channelling of arginine in NO and polyamine pathways in colonocytes and consequences. Front Biosci 16:1331–1343

Bower S, Kingdom J, Campbell S (1998) Objective and subjective assessment of abnormal uterine artery Doppler flow velocity waveforms. Ultrasound Obstet Gynecol 12:260–264

Budge H, Bispham J, Dandrea J et al (2000) Effect of maternal nutrition on brown adipose tissue and its prolactin receptor status in the fetal lamb. Pediatr Res 47:781–786

Buetow KC, Klein SW (1964) Effect of maintenance of "normal" skin temperature on survival of infants of low birth weight. Pediatrics 34:163–170

Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance. Physiol Rev 84:277–359

Chen CY, Carstens GE, Gilbert CD et al (2007) Dietary supplementation of high levels of saturated and monounsaturated fatty acids to ewes during late gestation reduces thermogenesis in newborn lambs by depressing fatty acid oxidation in perirenal brown adipose tissue. J Nutr 137:43–48

Clementi E, Nisoli E (2005) Nitric oxide and mitochondrial biogenesis: a key to long-term regulation of cellular metabolism. Comp Biochem Physiol A Mol Integr Physiol 142:102–110

Day RL, Caliguiri L, Kamenski C et al (1964) Body temperature and survival of premature infants. Pediatrics 34:171–181

de Boo HA, van Zijl PL, Smith DE et al (2005) Arginine and mixed amino acids increase protein accretion in the growth-restricted

and normal ovine fetus by different mechanisms. Pediatr Res 58:270–277
Delavaud C, Bocquier F, Chilliard Y et al (2000) Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. J Endocrinol 165:519–526
Dietz RE, Hall JB, Whittier WD et al (2003) Effects of feeding supplemental fat to beef cows on cold tolerance in newborn calves. J Anim Sci 81:885–894
Encinias HB, Lardy GP, Encinias AM et al (2004) High linoleic acid safflower seed supplementation for gestating ewes: effects on ewe performance, lamb survival, and brown fat stores. J Anim Sci 82:3654–3661
Folch J, Lees M, Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509
Fu WJ, Haynes TE, Kohli R et al (2005) Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr 135:714–721
Geng MM, Li TJ, Kong XF et al (2011) Reduced expression of intestinal *N*-acetylglutamate synthase in suckling piglets: a novel molecular mechanism for arginine as a nutritionally essential amino acid for neonates. Amino Acids 40:1513–1522
Glossmann H, Petrischor G, Bartsch G (1999) Molecular mechanisms of the effects of sildenafil (VIAGRA). Exp Gerontol 34:305–318
Gopalakrishnan G, Rhind SM, Stephenson T et al (2001) Effect of maternal nutrient restriction at defined periods in early to mid gestation on placento-fetal, kidney and adipose tissue weights at 110 days gestation in sheep. Early Hum Dev 63:58–59
Green AS, Rozance PJ, Limesand SW (2010) Consequences of a compromised intrauterine environment on islet function. J Endocrinol 205:211–224
Greenberg SS, Lancaster JR, Xie J et al (1997) Effects of NO synthase inhibitors, arginine-deficient diet, and amiloride in pregnant rats. Am J Physiol 273:R1031–R1045
Greenwood PL, Bell AW (2003) Consequences of intra-uterine growth retardation for postnatal growth, metabolism and pathophysiology. Reprod Suppl 61:195–206
Hales CN, Barker DJ (2001) The thrifty phenotype hypothesis. Br Med Bull 60:5–20
Jobgen W, Meininger CJ, Jobgen SC et al (2009) Dietary L-arginine supplementation reduces white fat gain and enhances skeletal muscle and brown fat masses in diet-induced obese rats. J Nutr 139:230–237
Knobel RB, Wimmer JE Jr, Holbert D (2005) Heat loss prevention for preterm infants in the delivery room. J Perinatol 25:304–308
Kwon H, Spencer TE, Bazer FW et al (2003) Developmental changes of amino acids in ovine fetal fluids. Biol Reprod 68:1813–1820
Lammoglia MA, Bellows RA, Grings EE et al (1999a) Effects of prepartum supplementary fat and muscle hypertrophy genotype on cold tolerance in newborn calves. J Anim Sci 77:2227–2233
Lammoglia MA, Bellows RA, Grings EE et al (1999b) Effects of feeding beef females supplemental fat during gestation on cold tolerance in newborn calves. J Anim Sci 77:824–834
Lang U, Baker RS, Braems G et al (2003) Uterine blood flow—a determinant of fetal growth. Eur J Obstet Gynecol Reprod Biol 110(Suppl 1):S55–S61
Lassala A, Bazer FW, Cudd TA et al (2010) Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J Nutr 140:1242–1248
Li X, Rezaei R, Li P et al (2011) Composition of amino acids in feed ingredients for animal diets. Amino Acids 40:1159–1168
Liu XD, Wu X, Yin YL et al. (2011) Effects of dietary L-arginine or *N*-carbamylglutamate supplementation during late gestation of sows on the miR-15b/16, miR-221/222, VEGFA and eNOS expression in umbilical vein. Amino Acids. doi:10.1007/s00726-011-0948-5
Mateo RD, Wu G, Bazer FW et al (2007) Dietary L-arginine supplementation enhances the reproductive performance of gilts. J Nutr 137:652–656
McKnight JR, Satterfield MC, Jobgen WS et al (2010) Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids 39:349–357
McMillen IC, Robinson JS (2005) Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 85:571–633
Moore RJ, Ong SS, Tyler DJ et al (2008) Spiral artery blood volume in normal pregnancies and those compromised by pre-eclampsia. NMR Biomed 21:376–380
National Research Council (NRC) (1985) Nutrient requirements of sheep. National Academy Press, Washington, DC
Nisoli E, Clementi E, Moncada S et al (2004a) Mitochondrial biogenesis as a cellular signaling framework. Biochem Pharmacol 67:1–15
Nisoli E, Falcone S, Tonello C et al (2004b) Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci USA 101:16507–16512
Reynolds LP, Redmer DA (2001) Angiogenesis in the placenta. Biol Reprod 64:1033–1040
Reynolds LP, Caton JS, Redmer DA et al (2006) Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol 572:51–58
Samson DE, Slee J (1981) Factors affecting resistance to induced body cooling in newborn lambs of 10 breeds. Anim Prod 33:59–65
Satterfield MC, Bazer FW, Spencer TE (2006) Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. Biol Reprod 75:289–296
Satterfield MC, Bazer FW, Spencer TE et al (2010) Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. J Nutr 140:251–258
Satterfield MC, McKnight JR, Li XL et al (2011) Nutrition, epigenetics, and vascular function. In: Maulik N (ed) Nutrition epigenetic mechanisms and human disease. CRC Press, New York, pp 125–139
Satterfield MC, Wu G (2011) Brown adipose tissue growth and development: significance and nutritional regulation. Front Biosci 16:1589–1608
Sell H, Deshaies Y, Richard D (2004) The brown adipocyte: update on its metabolic role. Int J Biochem Cell Biol 36:2098–2104
Simpson LL (1995) Sheep and lambs death loss. In: Washington: Livestock Section L, Dairy and Poultry Branch, Estimates Division, National Agriculture Statistics Service, USDA (ed)
Sladek SM, Magness RR, Conrad KP (1997) Nitric oxide and pregnancy. Am J Physiol 272:R441–R463
Symonds ME, Lomax MA (1992) Maternal and environmental influences on thermoregulation in the neonate. Proc Nutr Soc 51:165–172
Thaler I, Manor D, Itskovitz J et al (1990) Changes in uterine blood flow during human pregnancy. Am J Obstet Gynecol 162:121–125
Vosatka RJ, Hassoun PM, Harvey-Wilkes KB (1998) Dietary L-arginine prevents fetal growth restriction in rats. Am J Obstet Gynecol 178:242–246
Wang JJ, Wu ZL, Li DF et al (2011) Nutrition, epigenetics, and metabolic syndrome. Antioxid Redox Signal. doi:10.1089/ars.2011.4381
Wei JW, Carroll RJ, Harden KK et al (2011) Comparisons of treatment means when factors do not interact in two-factorial studies. Amino Acids. doi:10.1007/s00726-011-0924-0

Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1–17
Wu G (2010) Functional amino acids in growth, reproduction and health. Adv Nutr 1:31–37
Wu G, Knabe DA, Flynn NE et al (1996) Arginine degradation in developing porcine enterocytes. Am J Physiol Gastrointest Liver Physiol 271:G913–G919
Wu G, Davis PK, Flynn NE et al (1997) Endogenous synthesis of arginine plays an important role in maintaining arginine homeostasis in postweaning growing pigs. J Nutr 127:2342–2349
Wu G, Bazer FW, Cudd TA et al (2004) Maternal nutrition and fetal development. J Nutr 134:2169–2172
Wu G, Bazer FW, Wallace JM et al (2006) Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci 84:2316–2337
Wu G, Collins JK, Perkins-Veazie P et al (2007) Dietary supplementation with watermelon pomace juice enhances arginine availability and ameliorates the metabolic syndrome in Zucker diabetic fatty rats. J Nutr 137:2680–2685
Wu G, Bazer FW, Datta S et al (2008) Proline metabolism in the conceptus: implications for fetal growth and development. Amino Acids 35:691–702
Wu G, Bazer FW, Davis TA et al (2009) Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168
Wu G, Bazer FW, Burghardt RC et al (2010) Impacts of amino acid nutrition on pregnancy outcome in pigs: mechanisms and implications for swine production. J Anim Sci 88:E195–E204
Wu G, Bazer FW, Burghardt RC et al (2011) Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 40:1053–1063
Xiao XM, Li LP (2005) L-arginine treatment for asymmetric fetal growth restriction. Int J Gynaecol Obstet 88:15–18
Zeng X, Wang F, Fan X et al (2008) Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. J Nutr 138:1421–1425