THE ROLE OF MATERNAL PROTEIN INTAKE DURING LATE GESTATION ON PLACENTAL VASCULAR FUNCTION

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The role of maternal protein intake during late gestation on placental vascular function

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Global nutrient restriction or excess can influence umbilical hemodynamics in sheep fetuses (Chapter 2). We hypothesized that a specific component of the diet, namely maternal metabolizable protein (**MP**), would alter placental function. When MP restriction during late gestation occurs, we hypothesized that there would be a decrease in the sensitivity to bradykinin (**BK**) of the placental vascular arteries. In experiment 1, ewes received one of three isocaloric dietary treatments during late gestation: **MP60**: 60% of MP requirements; **MP80**: 80% of MP requirements; and **MP100**: 100% of the MP requirements on a dry matter basis from day 100 to 130 of gestation. In experiment 1, fetal and placental mass were not affected by dietary treatment; however, placental function was altered by a maternal diet low in protein. Ewes not meeting MP requirements during late gestation had fetal placental arteries that were more sensitive to **BK**-induced vasorelaxation; therefore we reject our hypothesis for experiment 1. In order to understand the mechanism of BK-induced vasodilation in the placental arteries, experiment 2 was designed. We hypothesized that MP level would alter the mechanism of BK-induced vasorelaxation in placental arteries. In experiment 2, ewes received one of three isocaloric dietary treatments during late gestation: **MP60**: 60% of MP requirements; **MP100**: 100% of the MP requirements; and **MP140**: 140% of MP requirements from day 100 to 130 of gestation. Maternal protein level during gestation did not impact the mechanism of BK-induced vasodilation; therefore we reject our hypothesis for experiment 2. However, the maternal and fetal placental vessels responded to BK through different mechanisms. In maternal placental arteries, pathways involving endothelium-derived hyperpolarizing factors (**EDHF**) and nitric oxide (**NO**) were responsible for BK-induced vasodilation, while the prostacyclin (**PGI2**) pathway did not greatly contribute to BK-induced vasodilation. The fetal placental arteries responded to BK through a mechanism that does not involve EDHF, NO, or PGI2, indicating that BK-induced vasorelaxation of the fetal placental arteries may be mediated through an unclassified EDHF-like pathway. It is important to realize the maternal and fetal placental arteries may respond to BK-induced vasodilation through different pathways when considering possible therapeutics for compromised pregnancies.

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Thank you to my parents for everything. You have always supported me, encouraged me, and believed in me. I would not be the person I am today without you. My successes as a whole are directly related to my parents - who I’ve become is a reflection of their love for me.

To my brother: jellyfish fist bumps and ma-niki-niki-naka! That’s my way of saying thank you for everything, and I love you. You are a fantastic person and have always been an inspiration to me.

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DEDICATION

“At the heart of science is an essential balance between two seemingly contradictory attitudes - an openness to new ideas, no matter how bizarre or counterintuitive they may be, and the most ruthless skeptical scrutiny of all ideas, old and new.” - Carl Sagan

“Sometimes I’ve believed as many as six impossible things before breakfast.” - Alice, Alice in Wonderland

To my parents, for always encouraging my imagination and wonder while also teaching me the art of skepticism. Thank you for opening doors and not closing others - you’ve made my journey into the wide, exciting worlds of science and teaching possible. I love you.

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LIST OF ABBREVIATIONS

AA.....amino acids

ACH ..... acetylcholine

ADF ..... acid detergent fiber

BCS.....body condition score

BF.....blood flow

BK.....bradykinin

BKR1..... bradykinin receptor 1

BKR2..... bradykinin receptor 2

BP..... blood pressure

℃ ..... degrees Celsius

CAR..... caruncular

CON ..... control

COT.....cotyledonary

COX-1 ..... cyclooxygenase-1

COX-2 ..... cyclooxygenase-2

CP ..... crude protein

CSA.....cross sectional area

DM.....dry matter

DRC.....dose response curve

E+.....endothelium-intact

E-..... endothelium-denuded

EC_{50}.....concentration of an agonist required to produce 50% of maximal response

EDV ..... end diastolic volume

eNOS.....endothelial nitric oxide synthase

g ..... grams

g/kg..... grams per kilogram

GUCY1B3.....soluble guanylate cyclase

HK .....high molecular weight kininogen

IBTX .....iberiotoxin

INDO .....indomethacin

IUGR ..... intrauterine growth restriction

KCl.....potassium chloride

kg/d.....kilograms per day

KKS ..... kallikrein kinin system

LK ..... low molecular weight kininogen

MA .....maternal artery

ml..... milliliters

mm .....millimeters

MMA ..... maternal mesenteric artery

MnV ..... mean velocity

MP .....metabolizable protein

MP100 ..... 100% metabolizable protein

MP140 ..... 140% metabolizable protein

MP60 ..... 60% metabolizable protein

MP80 ..... 80% metabolizable protein

N/A .....not applicable

NDF .....neutral detergent fiber

NE ..... net energy

NI ..... no inhibitor

NLA .....*N*(omega)-L-arginine methyl ester

NOS..... nitric oxide synthase

NRC..... National Research Council

NS ..... not significant

OMA ..... offspring mesenteric artery

OVR..... overnourished

pD_2 .....-log of the EC_{50} value

PE..... phenylephrine

PI .....pulsatility index

PSV ..... peak systolic velocity

RES ..... restricted

RI.....resistance index

SD .....standard deviation

SEM..... standard error of the mean

SNP .....sodium nitroprusside

U46619.....9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$

UA .....uterine artery

UmbA .....umbilical artery

UmbV ..... umbilical vein

UPLC..... ultra performance liquid chromatography

VEGF..... vascular endothelial growth factor

CHAPTER 1. LITERATURE REVIEW

Introduction

Developmental programming is the theory that a maternal insult or stimulus during any period of development, both *in utero* and during the neonatal period, can impair or enhance bodily systems and processes of the offspring, and that these impairments or enhancements early in life can have lasting effects on the offspring, even into adulthood (Godfrey and Barker, 2000). Epidemiologic evidence has identified maternal nutrition during pregnancy as a factor that affects fetal growth (Barker et al., 1993), and poor fetal development has been linked to several disorders including cardiovascular disease and hypertension in adult life (Barker et al., 1990; Barker et al., 1993).

Maternal nutrition may impact blood flow across the placenta, and adequate blood flow to the developing fetus is one of the most important factors for successful fetal development. Increasing uterine blood flow during the last half of gestation is critical for oxygen and nutrient delivery to the exponentially growing fetus, and alterations in placental blood flow or vasculature may compromise the growth of the developing fetus (Ford, 1995; Meschia, 1983; Redmer et al., 2004; Reynolds et al., 2005; Reynolds et al., 2006; Vonnahme et al., 2013). In normal pregnancies, uterine and umbilical blood flows increase exponentially throughout gestation (Reynolds et al., 1995); however, in models of intrauterine growth restriction (**IUGR**) caused by maternal undernutrition or overnutrition, blood flow to the fetus is reduced (Chandler et al., 1985; Wallace et al., 2001; Carr et al., 2012; Lemley et al., 2012).

An important question to consider is if these alterations to the health of the offspring, placenta, and placental blood flow are due to an overall caloric restriction or to restriction of a specific component in the diet. Evidence in rodent models suggests that maternal dietary protein level plays an important role in the health of the offspring. For example, maternal restriction of dietary protein results in offspring with glucose intolerance (Dahri et al., 1991; Langley et al., 1994), insulin resistance (Dahri et al., 1991), elevated blood pressure (Langley and Jackson, 1994; Ozaki et al., 2001), and vascular dysfunction (Itoh et al., 2002; Koumentaki et al., 2002; Torrens et al., 2002; Brawley et al., 2003).

While much has been learned about the role of maternal protein level and offspring health in rodents, this data cannot be directly applied to ruminants, and no data exist on the effects of maternal protein levels during gestation on the vascular function of the placenta in sheep. Because placental blood

flow is so important for normal fetal growth, and because maternal protein levels have been shown to alter vascular function, it is important to study how maternal protein level might impact the vascular function of the placental arteries.

This literature review will discuss: 1) developmental programming and the importance of protein in the maternal diet; 2) provide a brief overview on ovine placental development and the effect of maternal protein intake on placental development in ruminants; 3) detail the current literature available on the effects of maternal protein level during gestation on placental, fetal, and birth weight as well as hypertension in the offspring in both rodents and ruminants; 4) detail the effects of maternal protein level during gestation on the vascular function of maternal and offspring arteries; 5) provide a brief introduction to the kallikrein-kinin system and the likely importance of kallikrein and bradykinin in reproduction; 6) provide a brief overview on the role of arginine and nitric oxide in fetal growth and development; 7) discuss the use of non-invasive Doppler ultrasonography to measure the effects of maternal nutrition during late gestation on fetal and placental measurements and umbilical artery hemodynamics in sheep; 8) conclude with a statement of the problem that this dissertation will address.

This literature review will focus on studies involving ovine models; however, because of the limited data regarding maternal protein intake on vascular function in sheep, studies involving other species will be included as necessary. Following the literature review, four studies are reported. First, a study on the effects of maternal under- and over-nutrition on umbilical hemodynamics is presented. Following this are three studies on the effects of maternal metabolizable protein intake on fetal and placental measurements, blood flow to the fetus, amino acid concentrations in maternal and fetal circulation, and vascular function of the maternal and fetal placental arteries. This dissertation will then conclude with a general discussion and future directions.

Developmental programming and maternal protein level during gestation

Developmental programming is the idea that a maternal insult or stimulus during critical periods of development, both *in utero* and during the neonatal period, can impair bodily systems and processes of the offspring, and that these impairments early in life can have lasting effects on the offspring, even into adulthood (Godfrey and Barker, 2000). Data from epidemiologic studies suggests a link between

maternal nutrition during pregnancy and cardiovascular disease and hypertension of the offspring later in life (Barker et al., 1993; Barker and Clark, 1997).

Intrauterine growth restriction (**IUGR**) is often a result of suboptimal maternal nutrition during gestation. About 5% of human infants born in the United States suffer from IUGR (Marsal, 2002) and about 8% of human infants suffer from IUGR in other developed countries (Mandruzzato, et al., 2008), which can result in fetal and infant mortality and morbidity (Bernstein et al., 2000). Intrauterine growth restriction is also a major concern for the livestock industry since growth restriction during gestation causes negative impacts later in life on animal performance, including postnatal growth, body composition, and reproductive performance (reviewed in Wu et al., 2006). While IUGR is a significant problem for both humans and livestock species, the development of effective therapeutic options has not been possible because of the lack of knowledge about the mechanisms of IUGR. In order to investigate these mechanisms, extensive studies with multiple tissue collections need to be conducted, which is why developing animal models of IUGR is important for understanding exactly how IUGR is caused and how IUGR can lead to last health effects later in life.

Several ovine models exist that demonstrate the role of maternal undernutrition during gestation on placental and fetal growth (Charlton and Johengen, 1985; Holst et al., 1986, 1992; Faichney and White, 1987; McCrabb et al., 1991, 1992a, 1992b; Heasman et al, 1998; Bloomfield et al., 2000; Vonnahme et al., 2003; Scheaffer et al., 2004; McMullen et al., 2005; Lekatz et al., 2010a, 2010b; Lemley et al., 2012). An important question to consider is if these alterations to placental and fetal growth are due to an overall caloric restriction or to a specific component in the diet. Evidence in rodent models suggests that maternal dietary protein level plays an important role. The role of maternal protein intake during gestation on placental mass, fetal mass, birth weight, blood pressure, and vascular function will be discussed in greater detail later in this chapter, but briefly, maternal protein restriction can cause alterations to placental mass, fetal mass (Langley et al., 1996a, 1996b; Rees et al., 1999; Koumentaki et al., 2002), and birth weight (Langley et al., 1996a, 1996b) in rats. Further, maternal protein restriction can result in hypertension in the offspring (Brawley et al., 2003; Musha et al., 2006; Torrens et al., 2006) and decreased arterial sensitivity to endothelium-dependent vasorelaxants (Itoh et al., 2002; Koumentaki et al., 2002; Torrens et al., 2002; Brawley et al., 2003; Torrens et al., 2006). These data in rodents support

the theory that maternal protein restriction is one such insult that can alter the placenta and "program" the cardiovascular health of the offspring.

A review by Wu et al. (2004) suggests that maternal protein levels, particularly arginine levels, are important to placental and fetal growth. Figure 1.1 (adapted from Wu et al., 2004) briefly illustrates the hypothesized mechanism of how arginine affects fetal growth and development. When a dam is nutrient restricted, there will be less arginine that crosses the placenta and into fetal circulation (Casanello and Sobrevia, 2002). Further, arginine is a common precursor for nitric oxide (**NO**; Flynn et al., 2002), and IUGR is associated with impaired nitric oxide synthesis (Hata et al., 1998) and endothelial nitric oxide synthase (**eNOS**) activity (Casanello and Sobrevia, 2002). An overview of nitric oxide and its role in pregnancy is discussed later in this chapter, but briefly, nitric oxide aids in regulating placental angiogenesis, blood flow, and thus, the transfer of nutrients and oxygen from mother to fetus (Hefler et al., 2001; Bird et al., 2003), which ultimately impacts placental and fetal growth.

Because of the importance of arginine and nitric oxide to placental blood flow and nutrient transfer to the fetus, it stands to reason that a maternal diet that is low in protein may lead to negative effects on the placental and IUGR. Epidemiological studies in humans and animal studies in sheep link maternal undernutrition to alterations in placental mass, fetal growth, and birth weight. Studies in rodents suggest that maternal protein may play an important role in the growth and development of the fetus as well as in the health of the offspring later in life. Further, it has been hypothesized that maternal protein level, specifically arginine levels may alter placental function and blood flow to the fetus, which ultimately can lead to growth restriction and health issues later in life. Clearly, maternal protein level is important, but data regarding maternal protein intake during gestation are largely lacking in sheep. Therefore, studies utilizing an ovine model of maternal protein level are needed in order to understand how maternal protein during gestation may alter placental function.

Ovine placental development and the effect of maternal protein intake on placental development in ruminants

The ovine placenta is classified as cotyledonary and epitheliochorial (Senger, 2005). The endometrium of the sheep placenta contains two distinct areas, the aglandular caruncular region and the

highly glandular intercaruncular region (Ramsey, 1982). The caruncles, which are raised areas of the uterine endometrium, serve as the site for the implantation and placentation (Wimsatt, 1950; Amoroso, 1951). Placentation in the ovine involves the fusion of placental cotyledons with endometrial caruncles to form placentomes, which provide the site of exchange between the mother and developing fetus.

Early gestation is a critical time for the success of the pregnancy; however, because the focus of this dissertation is on late gestation, the many changes to the placenta that take place early in gestation will not be reviewed in this literature review. The ovine placenta grows rapidly early in pregnancy and then growth ceases during mid-gestation (Barcroft and Kennedy, 1939; Alexander, 1964; Stegeman, 1974). Placentome formation begins in the fourth week of gestation (Stegeman, 1974; Stevens, 1975). The number of placentomes in the gravid uterus vary, but 60 to 100 placentomes is normal (Alexander, 1964; Wimsatt, 1950). The total weight of the placentomes reaches a maximum between days 63 and 90 of gestation (Alexander, 1964; Stegeman, 1974). In the later stages of gestation, the total placentome weight decreases, mainly due to a decrease in the water content of the villi (Barcroft and Barron, 1946), which gradually disappears throughout gestation.

Few studies exist that examine the role of maternal protein intake in placental development in the ruminant. In a study by Amanlou et al. (2011), delivered placenta weight was heavier in ewes that were fed 114% of metabolizable protein requirements compared with ewes that were receiving 100% of metabolizable requirements during late gestation; however, there is no easily explainable reason why these results were observed since maximal placental weight is normally achieved by late gestation (Barcroft and Kennedy, 1939; Alexander, 1964; Stegeman, 1974). Sullivan et al. (2009) observed increased placental weight in heifers restricted to 75% of crude protein requirements during early gestation compared with heifers consuming 250% of crude protein requirements; however, Sullivan et al. (2009) also observed a decrease in placental weight compared to controls when the 75% crude protein diet was fed to a different breed of heifers, which suggests that breed may be a factor when considering how maternal protein intake affects placental development. Although differences in placental weight were observed between the 75% and 250% crude protein diets, there were no differences in cotyledonary weight, cotyledonary surface area, placental efficiency, or the number of caruncles (Sullivan et al., 2009).

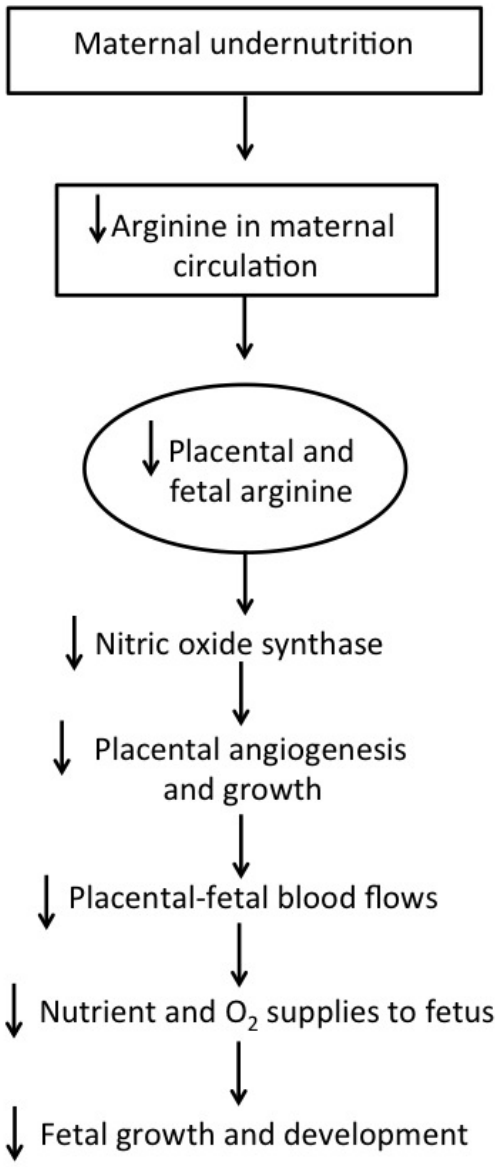

Figure 1.1. Schematic of the hypothesized mechanism of how maternal undernutrition, specifically maternal arginine restriction, impacts nitric oxide synthesis and, therefore, placental angiogenesis and placental blood, which can lead to reduced fetal growth and development. Adapted from Wu et al., 2004.

Studies designed to investigate how maternal protein intake during late gestation affects placental function in sheep, including vasoreactivity of placental arteries, nutrient transport across the placenta, and vascularity of the caruncular and cotyledonary tissues, are lacking. These studies would provide additional information on the role of maternal protein level on the placental development and function during late gestation when fetal growth is most rapid.

The effects of maternal protein level in the diet during gestation on placental, fetal, and birth weight and hypertension in the offspring

This section of the literature review will detail studies involving the effects of maternal protein level in the diet during gestation on placental, fetal, and birth weight in animal models. Because most research in this area has been done in rodents, studies utilizing rats will be discussed first followed by literature which exists for ruminants. Data regarding hypertension in the offspring is included when blood pressure of the offspring was measured.

Studies in rats

Because rodents provide genetic consistency in laboratory trials and have short gestation periods and a short average life span, researchers often use rodents as an experimental model. Rodents allow for research across multiple generations and throughout life, making them an attractive model for developmental programming experiments. There are quite a few rodent studies involving maternal protein level during gestation; for an overview of the select studies involving the effects of maternal protein level during gestation on placental and fetal measurements in rodents discussed in this literature review, please refer to Table 1.1.

Galler and Tonkiss (1991) were one of the first to investigate the effects of maternal protein level on the birth weight of pups in the rat. Male and female rats were fed diets containing either 25% or 6% of casein (by body weight) for 5 weeks before males and females on the same diet were mated. Pregnant female rats continued on their diet throughout gestation. The offspring from dams on the 6% casein diet were lighter at birth compared with offspring from the 25% dams (Galler and Tonkiss, 1991). According to the NRC (1995), the protein requirements for pregnant rats is 15% protein (by body weight); therefore,

Galler and Tonkiss did not truly have a control group, rather the study compared protein excess with protein restriction. Despite this, Galler and Tonkiss (1991) were one of the first to link maternal protein restriction with decreased birth weight.

As mentioned, the NRC (1995) recommends 15% protein (by weight) for pregnant rats; however, this is the minimum requirement, and from the rat studies covered in this literature review, it appears that a diet containing 18% protein is normal. It may be important to note that most of the studies reviewed in this chapter fed the 18% or 9% protein diets prior to mating. Since the NRC (1995) recommends 5% protein for maintenance in rats, these rats (especially ones on the 18% protein diet) were receiving excess protein during the periconceptional period.

Langley-Evans et al. (1994) conducted one of the first studies to investigate the association between maternal nutrition, fetal growth, and the later development of hypertension in the rat. Fourteen days prior to mating, rats were fed either a control (containing 18% casein by weight) or a restricted (9% casein by weight) diet. Diets continued through gestation (22 days). Maternal blood pressure was measured throughout pregnancy, pups were weighed at birth, and blood pressure in the offspring was measured at 4 weeks of age (Langley-Evan et al., 1994). Maternal blood pressure was unaffected by the low protein diet. Pups born to dams on the restricted protein diet weighed less at birth and had a higher blood pressure at 4 weeks of age compared to control offspring (Langley-Evans, et al., 1994). Next, to evaluate how a low protein diet might affect blood pressure in adult rats not exposed to protein restriction in utero, Langley et al. (1994) fed adult male and female rats either a control (containing 18% casein by weight) or a restricted (9% casein by weight) diet for 14 days, and found no differences in systolic blood pressure. This was one of the first studies to associate fetal exposure to maternal low protein diets with hypertension in offspring in rats.

After this study, Langley and Jackson (1994) designed a study to investigate differing levels of maternal protein in the diet. Female rats were fed diets containing either 18, 12, 9, or 6% of protein by weight from 14 days before breeding until delivery and found pups from the 6% group weighed less at birth than pups from any other treatment group. Further, the offspring from the 6% protein dams were lighter than offspring from the 18% protein dams from week 1 until week 21 of life. Mean offspring

Table 1.1. An overview of select studies in rats that include maternal protein restriction during gestation and the effects on placental weight, fetal weight, and birth weight.

Authors	Treatments[1]	Timing of treatments	Day(s) of assessment	Placental weight	Fetal weight	Birth weight
Brawley et al., 2003	18% casein (C) vs. 9% (R) casein	Day 0 to birth	Birth	N/A[2]	N/A	NS[3]
Guzman et al., 2006	20% casein (C) vs. 10% caseing (R)	Day 0 to birth	Birth	N/A	N/A	↓ R
Itoh et al., 2002	18% casein (C) vs. 9% (R) casein	Day 0 to 19	Day 19	NS	NS	N/A
Koumentaki et al., 2002	18% casein (C) vs. 9% (R) casein	Day 0 to 18	Day 18	↓ R	↓ R	N/A
Langley et al., 1996a	18% casein (C) vs. 9% (R) casein	Day -14 to day 12, 14, 16, 18, 20, or birth	Day 12, 14, 16, 18, 20, birth	12: NS 14: ↑ R 16: NS 18: NS 20: ↑ R Birth: N/A	12: NS 14: ↑ R 16: NS 18: NS 20: ↑ R	↓ R
Langley et al., 1996b	18% casein (C) vs. 9% (R) casein	Day -14 to 20, or birth	Day 20, birth	20: ↑ R Birth: N/A	20: ↑ R	↓ R
Musha et al., 2006	18% casein (C) vs. 9% (R) casein	Day 0 to birth	Day 175 of age	N/A	N/A	NS
Rees et al., 1999	18% casein (C) vs. 9% (R) casein	Day -14 to 19 or 20	Day 19, 20	19: NS 21: ↓ R	19: ↑ R 21: ↓ R	N/A
Torrens et al., 2002	18% casein (C) vs. 9% (R) casein	Day 0 to birth	Birth and day 19 of female offspring	Birth: N/A 19 (offspring): NS	NS	NS

Table 1.1. An overview of select studies in rats that include maternal protein restriction during gestation and the effects on placental weight, fetal weight, and birth weight (continued).

Authors	Treatments[1]	Timing of treatments	Day(s) of assessment	Placental weight	Fetal weight	Birth weight
Torrens et al., 2006	18% casein (C) vs. 9% (R) casein	Day 0 to 19 or birth	Day 19, birth	19: NS Birth: N/A	NS	NS

[1]Dietary treatments were diets formulated to provide either 18% protein (based on dam weight; **C** = control) or 9% protein (based on dam weight; **R** = restricted).
[2]N/A = not applicable; this measurement was not taken in the corresponding study
[3]NS = $P > 0.05$

systolic blood pressure of the dietary groups was observed to be inversely related to average maternal protein intake. The authors acknowledged that the diets used were not isoenergetic and stated the 6 and 9% protein diets were likely deficient in energy as well as protein (Langley and Jackson, 1994).

The need for isocaloric diets when investigating maternal protein restriction and hypertension in the offspring was demonstrated by Langley-Evans (2000). Pregnant rats were assigned to either a control protein diet or to a restricted protein diet. Further, each diet (control and restricted protein) was provided from one of two commercial operations and differed in fat content. Therefore the four diets were control protein-high fat, control protein-control fat, low protein-high fat, and low protein-control fat. Langley-Evans (2000) observed that offspring from the low protein dams were lighter at birth compared to offspring from dams fed control protein, regardless of fat level. However, hypertension was only observed in the low protein offspring compared with controls when dams were fed a high fat diet. Langley-Evans (2000) concluded that maternal protein level may influence birth weight, but fat content in the diet seems to contribute to hypertension in the offspring, and therefore, care should be taken to balance diets isocalorically and to make sure content of fat is similar between treatments.

Based upon the data of Langley and Jackson (1994), Langley-Evans et al. (1996a) chose to investigate an 18% protein diet (control) compared with a 9% protein diet (restricted), but care was taken to balance the diets calorically. The purpose of this study (Langley et al., 1996a) was to study how a protein restricted diet during gestation affects fetal growth throughout pregnancy. In this study (Langley et al., 1996a), the diets were fed from 14 days prior to breeding until day 12, 14, 16, 18, 20 of gestation or until birth, and placental and fetal/pup measurements were obtained at each time point. On days 14 and 20 of gestation, placental weight was greater in the restricted rats compared with the controls. The restricted fetuses were heavier compared to the controls at days 14, 18, and 20 of gestation, but had lower birth weights (average length of gestation = 22 days), which indicates that the fetuses from protein restricted dams did not experience the same growth rate during the last two days of gestation as fetuses from the control dams. Indeed, the data show fetuses in the control group increased body mass by 90% during

the last two days of gestation whereas fetuses in the restricted group only increased body mass by 70% during the same time period (Langley-Evans et al., 1996a). Further, total organ mass growth during the last two days of gestation was blunted in the fetuses from the restricted dams compared with fetuses from the control dams (Langley-Evans et al., 1996a). The reduction in body mass in the restricted offspring observed at birth was no longer present at 4 weeks of age. In fact, offspring from the protein restricted dams were heavier than control offspring at 4 weeks of age, and this increase in mass could not be explained by increases in liver, lung, heart, or brain mass. The blood pressure of the restricted offspring was higher at 4 weeks of age compared with the control offspring (Langley-Evans et al., 1996a). This was the first study in rats to show that protein restriction in the maternal diet may lead to increased growth throughout early and mid gestation, but this rate of growth is not sustained during rapid exponential growth of the fetus before delivery. Further, despite being lighter at birth, by 4 weeks of age, offspring of protein-restricted dams were heavier and were hypertensive compared to control offspring (Langley-Evans et al., 1996a).

The fetal growth observation was repeated by Rees et al. (1999) who fed female rats the same experimental diets as the Langley-Evans studies (i.e. 18% vs. 9% casein) from 14 days before mating until either day 19 or 21 of gestation. Maternal protein restriction increased fetal weight at day 19 of gestation, but by day 21 of gestation, fetuses from restricted dams weighed less compared to the controls. The data indicate the control fetuses experienced a 123% increase in mass from day 19 to 21 of gestation compared with only a 78% increase in mass in the restricted fetuses over the same time period (Rees et al., 1999). In this experiment (Rees et al., 1999), placental weight was not different between the two groups at day 19 of gestation, but by day 21, placental mass was decreased in the restricted animals.

Langley-Evans et al. (1996b) again reported that a 9% casein diet from 14 days prior to breeding through day 20 or delivery resulted in increased placental and fetal mass at day 20 of gestation, but reduced birth weight compared with control animals. At 7 weeks of age, offspring blood pressure was elevated in the restricted group compared with the control group (Langley-Evans et al., 1996b).

While maternal protein restriction increased fetal weight compared to controls at days 18, 29, and 20 in some studies (Langley-Evans et al., 1996a, 1996b; Rees et al., 1999), Koumentaki et al. (2002) observed decreased fetal and placental weight at day 18 of gestation in protein restricted animals compared with controls. The authors acknowledge that the protein restricted fetuses in this study (Koumentaki et al., 2002) experienced reduced mass earlier than fetuses from previously published data (Langley-Evans et al., 1996a, 1996b; Rees et al., 1999), but did not offer a hypothesis as to why this was observed. Itoh et al. (2002) and Torrens et al. (2006) did not see any differences at day 19 of gestation in fetal or placental mass in dams that were protein restricted throughout gestation. Langley-Evans et al. (1996a, 1996b) and Rees et al. (1999) utilized 56, 136, and 38 dams, respectively, while Koumentaki (2002), Itoh (2002), and Torrens et al. (2006) utilized 20, 21, and 34 dams, respectively. Perhaps if a larger sample size were studied, the fetal weight data would have been similar to the previously reported studies.

Brawley et al. (2003), Musha et al. (2006), and Torrens et al. (2006) all fed pregnant rats either 18% or 9% casein from day 0 of gestation until delivery. Birth weight was not affected by maternal protein level in any of the studies (Brawley et al., 2003; Musha et al., 2006; Torrens et al., 2006), but blood pressure was elevated in all offspring from protein restricted dams at 15 weeks of age (Torrens et al., 2006), male offspring from protein restricted dams at day 130 of age (Brawley et al., 2003) and in female offspring from protein restricted dams at day 175 of age (Musha et al., 2006).

Guzmán et al. (2006) fed rats either a 20% or a 10% casein diet from day 0 of gestation through weaning at day 21 of age. Female offspring from the protein restricted dams were lighter at birth compared with female offspring from control dams. This body weight difference persisted through weaning (day 21 of age) and into early adulthood (d 70 of age), but was no longer present in late adulthood (22 months of age).

The studies regarding the effects of maternal protein restriction on placental, fetal, and birth weight and offspring hypertension presented in this literature review indicate that when maternal protein intake is restricted, fetuses may experience faster growth up until day 20 of gestation, but during the last two days of pregnancy, which coincides with rapid, exponential fetal

growth in the rat, protein-restricted fetuses do not experience as great of growth as control fetuses; however, this pattern was not observed in all studies discussed. None of the studies discussed found that maternal protein restriction increases birth weight in offspring; birth weight was either not affected or was decreased with maternal protein restriction. Also, some of the studies discussed highlighted the importance for isocaloric dietary treatments with similar levels of all components except protein.

Studies in ruminants

There are a limited number of studies investigating the effects of maternal protein levels in the diet on placental and fetal growth and offspring weight in ruminants, and data regarding maternal protein intake during gestation on vascular function is nonexistent. In beef cattle, dam body weight and body condition score have been improved by late gestation crude protein supplementation (Anthony et al., 1986; Stalker et al., 2006; Funston et al., 2010), and these improvements may also enhance fetal growth and development. However, serial fetal measurements following maternal protein restriction during late gestation in cattle do not currently exist to my knowledge.

Micke et al. (2010) fed pregnant heifers a diet that was high in both metabolizable energy and crude protein or a diet that was low in both metabolizable energy and crude protein for the first trimester of pregnancy. During the second trimester, half of the heifers on each diet were switched to the opposite diet, resulting in four dietary treatments: HH (high metabolizable energy and high crude protein for both the first and second trimester), HL (high metabolizable energy and high crude protein for the first trimester followed by low metabolizable energy and low crude protein for the second trimester), LH (low metabolizable energy and low crude protein for the first semester followed by high metabolizable energy and high crude protein for the second semester), and LL (low metabolizable energy and low crude protein for the first trimester followed by low metabolizable energy and low crude protein for the second trimester). All heifers received similar diets during late gestation (Micke et al., 2010). Fetuses from heifers on the low metabolizable energy and low crude protein diet during the first trimester had a decreased curved crown rump

length at day 39 of gestation compared to fetuses from heifers receiving the high metabolizable energy and high crude protein diet, but this was not observed at day 68 or 95 of gestation (Micke et al., 2010). At day 95 of gestation, the umbilical cord diameter was decreased in the low metabolizable energy and low crude protein heifers compared with the high metabolizable energy and high crude protein heifers (Micke et al., 2010), suggesting that perhaps umbilical blood flow and nutrient delivery to the fetus were blunted in the low group. Calf birth weight was lower in heifers that were on the low metabolizable energy and low crude protein diet throughout the first and second trimesters compared with calves from heifers on the high metabolizable energy and high crude protein diet throughout the first and second trimesters (Micke et al., 2010). While this data is interesting, it is complicated by the fact that both metabolizable energy and crude protein levels were studied. Further, these dietary treatments were administered during the first two trimesters of gestation and not during the last third of gestation when fetal growth is most rapid. So while the data of Micke et al. (2010) does provide some insight into the effects of maternal protein levels on fetal growth in ruminants, further studies are needed to better investigate the role of maternal protein in ruminant fetal development.

The majority of studies involving crude protein supplementation during late gestation in cattle have found that calf birth weight is not significantly altered by crude protein supplementation. Anthony et al. (1986), Bohnert et al. (2002), Stalker et al. (2006), Martin et al. (2007), Larson et al. (2009), and Funston et al. (2010) all reported similar calf weights between control and crude protein supplemented dams during late gestation.

There are even fewer studies investigating maternal protein level during late gestation in the ewe. Ocak et al. (2005) fed pregnant ewes a control diet or a diet that was formulated to provide similar energy but 1.4 times the crude protein requirements from day 85 of gestation to lambing. Lamb birth weight was significantly increased in ewes receiving the high protein diet compared with ewes on the control diet (Ocak et al., 2005). In a study by Amanlou et al. (2011) investigating the effect of metabolizable protein supplementation, pregnant ewes were fed one of three isocaloric diets for the three weeks prior to lambing: a control diet, formulated to meet protein requirements, a moderate protein supplementation diet, formulated to provide a 14%

increase in protein requirements, or a high protein supplementation diet, formulated to provide a 24% increase in protein requirements. Maternal protein level did not alter lamb birth weight (Amanlou et al., 2011). Delivered placental weight was similar between ewes on the control and high protein diets and the delivered placenta from ewes on the moderate protein diet were heavier than placentas from the controls and high protein ewes (Amanlou et al., 2011).

Studies utilizing ruminants for the investigation of the role of maternal protein intake during late gestation are largely lacking, and the existing studies vary in design, diet formulation, and type of protein studied. Because of the significance of maternal protein intake on placental and fetal measurements, offspring hypertension, and vascular function in rodents (discussed below), additional studies are warranted in ruminants.

The effects of maternal protein level during gestation on vascular function in maternal and offspring arteries

Pregnancy is a unique ephemeral state with dramatic alterations in many physiological systems, including the vascular system (reviewed in Magness, 1998). During normal pregnancy these physiological changes are important for normal placental and fetal development to produce healthy offspring. There are numerous alterations to ensure adequate blood and thus supply of nutrients reaches the developing fetus including dramatic reductions in systemic vascular resistance and increases in cardiac output, heart rate, stroke volume, and blood volume; as peripheral resistance falls, blood pressure is maintained by a rise in cardiac output (Ford, 1982; Rosenfeld, 1984; Magness and Zheng, 1996, Magness, 1998). Compared to all other vascular beds, the uteroplacental vascular bed undergoes the most dramatic cardiovascular alterations during gestation.

Endothelial cells are involved in the control of vascular tone through the release of nitric oxide (Furchgott and Zawadzki, 1980; Ignarro et al., 1981), prostacyclin (Moncada et al., 1976), and endothelium-derived hyperpolarizing factor (Nagao and Vanhoutte, 1993). Vascular defects to the maternal and offspring vasculature beds such as endothelial dysfunction may be caused by maternal protein restriction during gestation. This section of the literature review will address

studies that investigate the effects of maternal protein restriction during gestation on vascular function both in the dams and in the offspring, and will be limited to the effects on endothelium-dependent vasorelaxants. When experiments to study the mechanisms contributing to endothelium-dependent vasorelaxants were performed, these results will be included as well. Because, currently, the effects of maternal protein level on vascular function are largely lacking for ruminant species, this literature review will cover studies in rats, where detailed experiments have been conducted on this subject. For an overview of the select studies involving the effects of maternal protein level on vascular function in rats, please refer to Table 1.2.

Koumentaki et al. (2002) hypothesized that initial vasodilation in pregnant rats is likely to involve much of the systemic circulation and that peripheral vasodilatation would be reduced in rats fed a low-protein diet during pregnancy. To test this hypothesis, twenty pregnant rats were fed either a diet containing 18% casein (by body weight) or a diet containing 9% casein (by body weight) from day 0 until day 18 of gestation. At this time, cumulative concentration-response curves to the endothelium-dependent vasorelaxant acetylcholine (**ACh**) were obtained in the mesenteric artery of the pregnant dams. The sensitivity of mesenteric arteries to ACh was attenuated in dams fed the protein restricted diet compared with the control diet, which supports the original hypothesis. To investigate which pathway might be responsible for this endothelium-dependent relaxation, dose response curves to ACh were repeated following incubation with a combination of *N*-nitro-L-arginine methyl ester (**NLA**) and indomethacin (**INDO**), which inhibit nitric oxide synthase and cyclooxygenase, respectively. The maximal response to ACh following incubation with the inhibitors was not different from the ACh dose response curve without inhibitors in the control animals, suggesting that vasodilation of the mesenteric arteries in the control animals is occurring through a pathway not involving nitric oxide or prostacyclin. However, in the protein restricted animals, the maximal response to ACh was blunted by 48% following incubation with the inhibitors, which indicates in the restricted animals, one or both of the nitric oxide and prostacyclin pathways is mediating mesenteric arterial vasodilation in response to ACh (Koumentaki et al., 2002).

Next, a study was designed to investigate if maternal protein restriction during gestation altered the vasoreactivity of the uterine artery (Itoh et al., 2002). Similar to Koumentaki et al. (2002), pregnant rats were fed either the 18% protein diet or the 9% protein diet from day 0 until day 18 of gestation. Uterine arteries were collected and used for dose response curves to ACh. In this study (Itoh et al., 2002), maternal protein restriction did not blunt the sensitivity to ACh compared to the control animals. In order to determine if the mechanism driving ACh-induced vasorelaxation in the uterine artery involved the nitric oxide pathway, the dose response curve was repeated after incubation with NLA, and in both the treatment groups, the maximal response to ACh was reduced, which indicates nitric oxide is partly responsible for ACh-induced vasodilation of the uterine artery (Itoh et al., 2002). Itoh et al. (2002) also studied the vasodilatory effects of VEGF on the uterine artery and found that VEGF did induce vasodilation of the uterine artery in both treatment groups, but that the sensitivity and maximal response to VEGF was decreased in the protein restricted group. The VEGF dose response curves were repeated in the presence of NLA, INDO and the combination of the two. In the 18% casein group, the maximal response to VEGF was significantly reduced by INDO alone and further reduced by the combination of NLA and INDO, but in the 9% casein group, the maximal response to VEGF was reduced by INDO, but was not further reduced by the combination of NLA and INDO (Itoh et al., 2002). These results indicate that in control fed animals, both the nitric oxide and prostacyclin pathways are important for VEGF-mediated vasorelaxation of the uterine artery, but in the protein restricted group, the prostacyclin pathway appears to mediate VEGF-mediate relaxation more so than the nitric oxide pathway.

Table 1.2. An overview of select studies in rats that include maternal protein restriction during gestation and the effects on vascular function of either maternal or offspring arteries.

Authors	Treatments[1]	Timing of treatments	Day(s) of assessment	Vessel(s) analyzed	Endothelium-dependent vasodilator(s)	Summary of results
Brawley et al., 2003	18% casein (C) vs. 9% (R) casein	Day 0 to birth	DRC[2]: Day 87, 164 of age BP: Day 130 of age	OMA[3]	ACh[4] BK[5]	ACh: ↓ R at days 87 and 164 BK ↓ R at days 87 and 164 BP[6]: ↑ R
Itoh et al., 2002	18% casein (C) vs. 9% (R) casein	Day 0 to 19	Day 19	UA[7] OMA	ACh VEGF[8]	UA: ACh: NS[9] VEGF: ↓ R OMA: ACh: N/A[10] VEGF: NS
Koumentaki et al., 2002	18% casein (C) vs. 9% (R) casein	Day 0 to 18	Day 18	MMA[11]	ACh	ACh: ↓ R
Koumentaki et al., 2002	18% casein (C) vs. 9% (R) casein	Virgin rats, fed for 18 days	18 days after diets initiated	MMA	ACh	ACh: ↓ R
Musha et al., 2006	18% casein (C) vs. 9% (R) casein	Day 0 to birth	DRC: Day 175 of age BP: Day 175 of age	OMA	ACh BK	ACh: NS BK: NS BP: ↑ R
Torrens et al., 2002	18% casein (C) vs. 9% (R) casein	Day 0 to birth	Day 19 of female offspring	MMA	ACh	ACh: ↓ R

Table 1.2. An overview of select studies in rats that include maternal protein restriction during gestation and the effects on vascular function of either maternal or offspring arteries (continued).

Authors	Treatments[1]	Timing of treatments	Day(s) of assessment	Vessel(s) analyzed	Endothelium-dependent vasodilator(s)	Summary of results
Torrens et al., 2006	18% casein (C) vs. 9% (R) casein	Day 0 to 19 or birth	DRC: Day 19, birth BP: Day 105 of age	UA OMA	ACh VEGF	UA: ACh: ↓ R OMA: ACh: NS VEGF: ↓ R BP: ↑ R

[1]Dietary treatments were diets formulated to provide either 18% protein (based on dam weight; **C** = control) or 9% protein (based on dam weight; **R** = restricted).
[2]DRC = dose response curve
[3]OMA = offspring mesenteric artery
[4]ACh = acetylcholine
[5]BK = bradykinin
[6]BP = blood pressure
[7]UA = uterine artery
[8]VEGF = vascular endothelial growth factor
[9]NS = $P > 0.05$
[10]N/A = not applicable; this measurement was not taken in the corresponding study
[11]MMA = maternal mesenteric artery

Because maternal protein restriction in pregnant rats has consistently resulted in hypertension in the offspring (Langley-Evans et al., 1994; 1996a; 1996c; Gardner et al., 1997), Brawley et al. (2003), Torrens et al. (2002; 2006), and Musha et al. (2006) began investigations of vascular function on the offspring from protein restricted dams. In order to test the hypothesis that exposure to a protein-restricted diet *in utero* may induce vascular defects in the resistance arteries of adult offspring (which may play a part in the maintenance of the elevated systolic blood pressure observed in offspring of this rodent model), pregnant rats were fed a diet containing either 18% casein or 9% casein from day 0 to delivery (Brawley et al., 2003). Male offspring from this study had increased blood pressure when exposed to low protein *in utero* compared with male offspring from control fed dams (Brawley et al., 2003). The mesenteric arteries from male offspring from these mothers were then analyzed at day 87 or day 164 of age for the response to the endothelium-dependent vasorelaxants ACh and bradykinin (BK). At day 87 of age, the overall sensitivity of mesenteric arteries to ACh was attenuated in the male offspring from protein restricted dams, but the maximal response was similar between the males from control and protein restricted dams. Also at day 87 of age, the overall sensitivity and the maximal response of mesenteric arteries was blunted in males from protein restricted animals compared with offspring from control mothers (Brawley et al., 2003). By day 164 of age, both the sensitivity and the maximal response of the mesenteric arteries to ACh and BK were attenuated in male offspring from protein restricted mothers (Brawley et al., 2003). Brawley et al. (2003) was the first to show that mesenteric arterial function in response to endothelium-dependent vasorelaxants is impaired in rats exposed *in utero* to maternal low protein diets; therefore, dietary protein restriction in pregnancy induces vascular defects in isolated arteries, which results in hypertension of the offspring (Brawley et al., 2003).

Because male offspring subjected to low protein *in utero* were hypertensive and had impaired vascular function (Brawley et al., 2003), Torrens et al. (2002) hypothesized that female offspring exposed to low protein *in utero* would have impaired vascular function during pregnancy despite protein restriction outside of the uterus. To test this hypothesis, pregnant rats were fed diets with either 18% casein or 9% casein from day 0 until delivery. Birth weight of the female

dams did not differ (Torrens et al., 2002) and the female offspring received a control diet throughout life. Female offspring were mated, and on day 19 of gestation the mesenteric arteries of the now pregnant female offspring were collected, and the dose response curve to ACh was obtained. Mesenteric arteries from pregnant female offspring exposed to low protein *in utero* were less sensitive to ACh than mesenteric arteries from female offspring of control dams (Torrens et al., 2006). These results were the first to associate low protein *in utero* to impaired vascular function in pregnant female offspring (Torrens et al., 2002), and indicate that protein restriction during gestation does program the cardiovascular health of female offspring later in life.

The effect of *in utero* protein restriction due to maternal diet on the vascular function of mesenteric arteries in response to ACh and BK was also investigated in nonpregnant female offspring (Musha et al., 2006). Dams were fed a diet containing either 18% casein or 9% casein from day 0 until delivery and female offspring were raised on a control diet throughout life. By day 175 of age, blood pressure was increased in female offspring subjected to low protein during fetal development compared with control offspring (Musha et al., 2006). In this study, the mesenteric arterial response at day 175 of age to both ACh and BK was not different between female offspring from control and protein restricted dams (Musha et al., 2006). Musha et al. (2006) recorded female offspring blood pressure from day 50 to day 175 of life, and blood pressures were similar between females from control dams and protein restricted dams until day 175 of age. Perhaps differences in the endothelium-dependent relaxant dose response curves were not observed at day 175 of age because these female offspring were just beginning to develop hypertension compared to the control offspring.

Another study by Torrens et al. (2006) investigated the effect of maternal protein restriction on both the uterine artery of pregnant dams and of the mesenteric arteries of male offspring. Pregnant rats were again fed a diet with either 18% casein or 9% casein from day 0 until either day 19 of gestation or delivery. At day 19, maternal uterine arteries were collected for vasoactive studies in response to both ACh and VEGF. The sensitivity of the uterine artery to ACh did not differ between controls and protein restricted animals (Torrens et al., 2006), but the maximal response of the uterine artery to VEGF was attenuated in the protein restricted dams

compared with controls (Torrens et al., 2006). In order to investigate the mechanism of VEGF-induced relaxation in the uterine artery, vessels were incubated with NLA, INDO or the combination of the two. In the presence of NLA, uterine arteries reached maximal relaxation, indicating that nitric oxide is not responsible for VEGF-induced relaxation of uterine arteries in either dietary treatment group. Rather, Torrens et al. (2006) reports that prostacyclin is likely responsible for VEGF-induced relaxation of the uterine artery since incubation with INDO blunted uterine arterial response to VEGF. The addition of NLA to INDO did not change the uterine artery response compared to INDO alone, again confirming that prostacyclin contributes to VEGF-induced relaxation in the uterine artery more so than nitric oxide (Torrens et al., 2006).

In the offspring from this study (Torrens et al., 2006), mesenteric arterial response at 15 weeks of age to ACh was attenuated in males exposed to amaternal low protein diet *in utero* compared to males exposed to the control maternal diet *in utero*. The offspring mesenteric arteries were only subjected to a combination of NLA and INDO rather than the two inhibitors separately. Inhibition of both nitric oxide synthase and cyclooxygenase significantly impaired the mesenteric arterial response to ACh in males exposed to the maternal control diet *in utero*, but inhibition of these pathways in males subjected to protein restriction in utero had no effect on the mesenteric arterial response to ACh (Torrens et al., 2006). This suggests that when exposed to a maternal diet low in protein during fetal development, the mesenteric arteries of offspring are programmed to respond to endothelium-dependent relaxants via a pathway independent of nitric oxide or prostacyclin.

Overall, these studies indicate that maternal protein restriction during pregnancy impairs the vascular function of both maternal and offspring arteries in response to endothelium-dependent vasorelaxants. When attempting to determine the mechanism of endothelium-dependent vasorelaxation, the overall results are conflicting. It appears in some cases maternal dietary protein intake influences the mechanism of endothelium-dependent vasodilation and not in others. Further, the data indicate that endothelium-dependent responses in the vessels analyzed (maternal mesenteric, uterine artery, offspring mesenteric) may be mediated by different

mechanisms. Further studies are needed to better understand how endothelium-dependent vasorelaxants are eliciting vascular responses.

The kallikrein-kinin system

The scientific interest in the kallikrein-kinin system (**KKS**) can partly be explained by the duality and the complexity of this system. The KKS includes two kininogens [high molecular weight kininogen (**HK**) and low molecular weight kininogen (**LK**)], two kallikreins (plasma kallikrein and tissue kallikrein), two main kinins [bradykinin and kallidin), two receptors (bradykinin receptor 1 (**BKR1**) and bradykinin receptor 2 (**BKR2**)], and two kinin-forming systems (plasma kallikrein-kinin system and tissue kallikrein-kinin forming system). The duality of the KKS extends to the pharmacological activities of the system: the kinins of the KKS can result in either vasoconstriction or vasodilation. The complexity of the KKS is evident by the presence of the different constituents in plasma and numerous tissues throughout the body. Further, the KKS has multiple relationships with other important physiologic pathways such as the renin-angiotensin and coagulation pathways. For the purpose of this literature, most components of the KKS will only be briefly described, and the focus will mainly be on the kinin bradykinin.

Briefly, the KKS represents a cascade, that when activated, triggers the release of active kinins from kininogens by kallikrein enzymes. These kinins then exert their pharmacological activities by binding to specific receptors before being metabolized by various peptidases (reviewed in Moreau et al., 2005).

Kininogens

High molecular weight kininogen (**HK**) and low molecular weight kininogen (**LK**) are the precursors of kinins, and are encoded by the same gene but then undergo alternate splicing (Kitamura et al., 1985; Takagaki et al., 1985). The HK circulates in human plasma as an 88- to 120-kDa single-chain glycoprotein at a concentration of 70 to 90 μg/mL (Adam et al., 1985). The LK ranges from 50 to 68 kDa and circulates in human plasma at a concentration of 170 and 220 μg/mL (Muller-Esterl et al., 1982; Adam et al., 1985).

The kinin-forming systems

A simplified illustration of the kinin-forming system is shown in Figure 1.2. There are two main pathways by which kinins are generated. The plasma kallikrein-kinin system is the more complex pathway, and initiates activation of the intrinsic coagulation pathway. This pathway involves HK as the substrate for plasma kallikrein to release bradykinin. Plasma kallikrein is encoded by a single gene and is synthesized in the liver (Asakai et al., 1897; Yu et al., 1998). It is secreted as inactive prekallikrein, which binds to HK (Mandle et al., 1976; Reddigari and Kaplan, 1989; reviewed in Bhoola et al., 1992). Contact of plasma with a negatively charged surface leads to the binding and autoactivation of factor XIII to factor XIa, activation of prekallikrein to kallikrein, and cleavage of HK by kallikrein to release bradykinin (Mori et al., 1981; Kaplan et al, 1997). Another mechanism for the initiation and activation of the KKS involves binding of HK to the surface of cells such as leukocytes, platelets, and endothelial cells (Zhao et al., 2001). Binding of HK to endothelial cells activates prekallikrein to kallikrein and release of bradykinin from HK (Nishikawa et al., 1992; Motta et al., 1998; Lin et al., 2000; Zhao et al., 2001).

Tissue kallikreins are members of a large multigene family (Diamandis et al., 2000), of which only one gene, *KLK1*, encodes for an enzyme capable of releasing a vasoactive kinin (Clements, 1997; Margolius, 1998; Diamandis et al., 2000). For the purposes of this review, the term “tissue kallikrein” will refer to the product of *KLK1*, the kallikrein with the ability to release active kinins from kininogen precursors. Tissue kallikrein is widely distributed in a number of tissues including the kidney, central nervous system, pancreas, gut, salivary glands, spleen, adrenal glands, and blood vessels (reviewed in Bhoola et al., 1992; Mahabeer and Bhoola, 2000). Tissue kallikrein is synthesized as a prokallikrein, and trypsin, plasmin, activated plasminogen (Yamade &Erdös, 1982), and plasma kallikrein (Takada et al., 1985) have all been shown to cleave prokallikrein to the active tissue kallikrein *in vitro*. Tissue kallikrein releases the vasoactive kallidin from LK, and although LK is considered the main substrate of tissue kallikrein, this enzyme is also capable of cleaving HK to release bradykinin (reviewed in Mahabeer and Bhoola, 2000).

Kinin receptors

There are two G-protein-coupled receptors that mediate the cellular effects of the kinins, the BKR1 and BKR2 receptors (Leeb-Lundberg et al., 2005). Because both BKR1 and BKR2 are G-protein-coupled receptors, they are similar in structure with a single polypeptide chain that spans the membrane seven times, with the amino

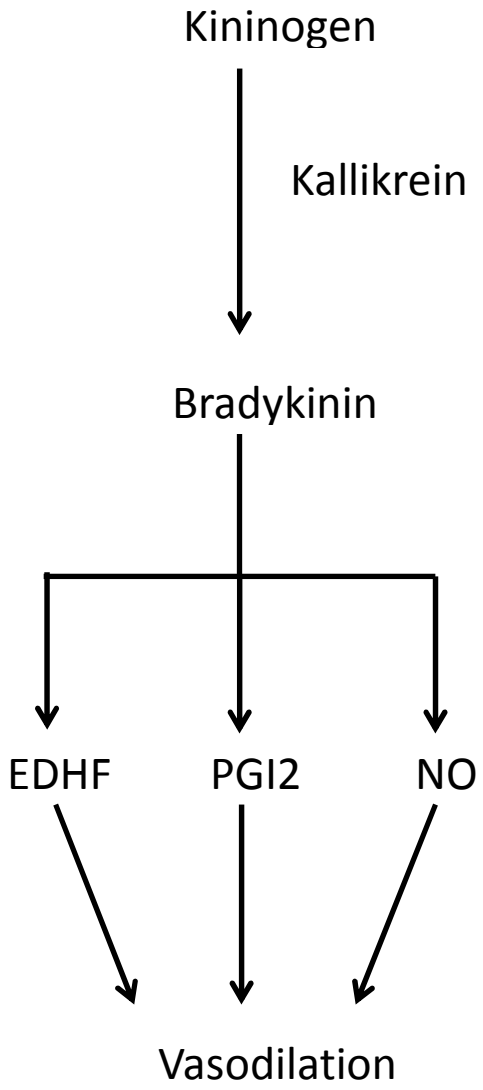

Figure 1.2. A simplified illustration of the kallikrein-kinin system pathway. Kininogen is cleaved by the enzyme kallikrein to form bradykinin, a potent vasodilator. Bradykinin elicits vasodilation through one of three classic pathways: endothelial-derived hyperpolarizing factor (**EDHF**), prostacyclin (**PGI2**), or nitric oxide (**NO**).

terminus being extracellular and the carboxyterminus being intracellular. Despite the similar structure, the two proteins are expressed very differently. Numerous studies have consistently shown BKR2 is present in most tissues, particularly in endothelial cells and smooth muscle cells, but BKR1 is not expressed in significant levels in normal tissues, rather BKR1 is inducible and expression of BKR1 is up-regulated following tissue injury (reviewed in Marceau et al., 1998; Christiansen et al., 2004; reviewed in Marceau and Regoli, 2004; Souza et al., 2004; reviewed in Moreau et al., 2005).

General pharmacology of bradykinin

Bradykinin is a nonapeptide, biologically active, naturally occurring vasoagent (Regoli and Barabé, 1980). Circulating levels of bradykinin are difficult to measure accurately since sampled blood contains the necessary components to generate and destroy these peptides *in vitro* and also because bradykinin is an autocoid that is only active close to its site of formation (Blais et al., 2000). The vasoresponses elicited by bradykinin are determined by the stimulation of endothelial cells from which secondary messengers are released under the influence of Ca^{2+}-sensitive cascades to influence the vascular smooth muscle. Upon bradykinin binding to BKR2, signal transduction activates several secondary messenger systems. Activation of adenylyl cyclase or guanylyl cyclase is a transduction mechanism that leads to the production of cAMP and cGMP, which is a known vasodilatory mechanism in vascular smooth muscle. Bradykinin-induced release of nitric oxide from the endothelial cells, and the subsequent rise of cGMP in smooth muscle cells, leading to vasorelaxation has been documented in a number of systems. Bradykinin also can lead to the release of prostacyclin from the endothelium cells, which will result in an increase in cAMP in smooth muscle cells, also leading to vasorelaxation. Other mechanisms of endothelium-dependent vasorelaxation have been documented including endothelium-dependent hyperpolarization of smooth muscle cells (Batenburg et al., 2004; reviewed in Marceau and Regoli, 2004; reviewed in Moreau et al., 2005).

The role of kallikrein and bradykinin in reproduction

Because tissue kallikrein mRNA expression was greatest during the proestrus stage of the estrous cycle in the rat (Corthorn and Valdés, 1994), Corthorn et al. (1997) hypothesized that kallikrein was stimulated by estrogen. In order to test this hypothesis, Corthorn et al. (1997) ovariectomized rats and assigned them to either a control group (no estrogen) or to a treated group that received subcutaneous doses of estrogen or progesterone. Uteri were collected at different days of supplementation and used for a tissue kallikrein assay. Tissue kallikrein expression was increased with estrogen supplementation but not with progesterone, thus Corthorn et al. (1997) concluded that estrogen stimulates kallikrein expression in the uterus, which explains the increases in kallikrein mRNA observed during high levels of estrogen during the estrous cycle (Corthorn and Valdés, 1994). Further, because uterine kallikrein expression was similar between the control and progesterone groups, Corthorn et al. (1997) concluded that progesterone does not stimulate kallikrein expression.

If kallikrein is stimulated by estrogen, and its expression is increased during the estrous cycle (Corthorn et al., 1997) as well as early pregnancy (Valdés et al., 1993), and if kallikrein releases the vasoactive peptide bradykinin (reviewed in Bhoola et al., 1992), it stands to reason that both kallikrein and bradykinin are important in pregnancy in rats. Further, because bradykinin is a potent vasodilator (reviewed in Bhoola et al., 1992), and because increasing blood flow is necessary for successful nutrient transfer to the fetus (Ford, 1995; Meschia, 1983; Redmer et al., 2004; Reynolds et al., 2005; Reynolds et al., 2006), it is reasonable to assume that kallikrein and bradykinin are factors contributing to the necessary increases in blood flow during gestation. Moreover, both tissue kallikrein and BKR2 have been localized to luminal and glandular epithelial cells in the rat (Valdés et al., 1993; Valdés et al., 1996; Corthorn and Valdés, 1997; Figueroa et al., 2001) and human uterus (Clements et al., 1994; Clements and Mukhtar, 1997; Figueroa et al., 1997), indicating a role for bradykinin in reproduction. Valdés et al. (2001) further addressed the role of kallikrein and bradykinin during gestation by evaluating the temporospatial pattern of kallikrein and BKR2 in the human uterus in early and late pregnancy and found kallikrein and

BKR2 in the luminal and glandular epithelium, decidual cells, trophoblast invading arteries, endothelium, and vascular and myometrial smooth muscle. Valdés et al. (2001) pointed out these are sites that compose the fetomaternal interface and intervene in the contact and attachment of the embryo, in placental development, and in parturition, and, therefore, concluded that the kallikrein-kinin system and bradykinin likely play a role in embryo attachment, implantation, placentation, and maintenance of placental blood flow through processes likely involving vasodilation, increased vasopermeability, and stimulation of cell proliferation.

The role of arginine and nitric oxide in fetal growth and development

The focus of this dissertation centers on maternal protein intake during late gestation on placental vascular function. Because Wu et al. (2004) hypothesized that arginine and nitric oxide, which is synthesized from arginine, aid in fetal growth and development, it can be hypothesized that a maternal diet low in protein (and therefore arginine) may impact fetal growth and development through mechanisms involving nitric oxide. Further, this dissertation addresses the action of bradykinin in placental arteries, and the nitric oxide pathway is one way bradykinin elicits vasodilation. Therefore, this section of the literature will address the topic of arginine and nitric oxide in fetal development in sheep.

Arginine is a precursor for nitric oxide via nitric oxide synthase (Flynn et al., 2002). Nitric oxide is a major endothelium-derived vasorelaxant and is important for regulating placental-fetal blood flows, and, therefore, nutrient transport to the fetus (Bird et al., 2003). Preventing nitric oxide synthesis, either by nitric oxide synthase inhibitors or by endothelial nitric oxide synthase knockout models in mice, results in IUGR (Hefler et al., 2001). Further, feeding arginine-free diets to pregnant rats or inhibiting nitric oxide synthesis resulted in increased fetal resorptions, IUGR, increased perinatal mortality, and decreased number of live fetuses (Greenberg et al., 1997), but when dietary arginine is supplemented to pregnant rats, fetal growth restriction is reversed (Vosatka et al., 1998). The results of Greenberg et al. (1997) indicate that endogenous synthesis of arginine is insufficient for pregnant dams, and arginine must be provided from diets to insure optimal fetal survival and growth.

The effects of a low protein diet during gestation on arginine concentrations and nitric oxide synthesis in ruminants has not been investigated, but feeding a low protein diet from mating until day 40 or 60 of gestation in pigs resulted in decreased arginine concentrations in fetal plasma and allantoic fluid (Wu et al., 1998a) and decreased placental nitric oxide synthase activity (Wu et al., 1998b). In sheep, a 50% global restriction of NRC requirements between day 28 and 78 of gestation decreased arginine concentrations in maternal plasma, fetal plasma, and allantoic fluid at day 78 in sheep (Kwon et al., 2004) while a 40% global restriction of NRC requirements between days 50 and 130 of gestation resulted in decreased arginine in maternal plasma in sheep at day 130 of gestation (Lekatz et al., 2011). Further, global maternal nutrient restriction in sheep from day 0 until day 70 of gestation increased arterial blood pressure in the ovine fetus and impaired nitric oxide-dependent vasodilation of fetal femoral arteries at day 127 of gestation (Ozaki, et al., 2000).

These studies indicate that maternal arginine intake during pregnancy is important for the growth and development of the fetus. Maternal diets lacking in arginine may impair fetal development or vascular function through mechanisms involving nitric oxide.

Using Doppler ultrasound to measure the effects of maternal nutrition during late gestation on fetal and placental biometry and umbilical artery hemodynamics in sheep

Historically, animal research has relied on multiple tissue collections in order to gather cross-sectional data of fetal and placental growth throughout gestation; however, a major limitation of this method is that each time point is derived from a different cohort of pregnancies, which may contribute variation to the experimental design. By contrast, ultrasonography offers a noninvasive means of assessing fetal growth longitudinally within a single cohort of pregnancies.

In clinical practice, umbilical artery Doppler waveform analysis is the primary tool for diagnosing and monitoring fetuses with IUGR (Baschat, 2010). Umbilical artery Doppler measurements provide a noninvasive method for evaluating umbilical blood flow and impedance

to blood flow, and UA indicies correlate well with directly measured blood flow and vascular resistance in normal sheep pregnancy (Maulik et al., 1989; Nimrod et al., 1989; Acharya et al., 2004).

The pulsatility index (**PI**) and resistance index (**RI**) are pulsed-wave Doppler measurements of downstream resistance in arteries. These indices normally decline as gestation progresses and correlate positively with measured umbilical vascular resistance and negatively with umbilical blood flow (Newnham et al., 1987; Wallace et al., 2001; Acharya et al., 2004). Doppler ultrasonography also allows for measurement of placentomes, fetal biparietal diameter, and fetal abdominal girth.

Very few studies have used noninvasive Doppler ultrasonography to measure umbilical artery PI and RI in sheep IUGR models induced by maternal under- or over-nutrition. For the purpose of this literature review, only two studies involving maternal nutrition during late gestation in sheep will be discussed. Although limited, the data available are in agreement that fetal growth measurements increase with advancing gestation, placentome size peaks around d 80 of gestation, and umbilical artery PI and RI decrease throughout gestation in normal pregnancies (Carr et al., 2012; Lemley et al., 2012).

In a well-established model of IUGR in an overnourished model (reviewed in Wallace et al., 2006), pregnant adolescent ewes received either a control diet, formulated to meet nutritional requirements or a high diet, formulated to exceed nutritional requirements from embryo transfer to day 131 of gestation. Noninvasive Doppler techniques were used to measure umbilical artery hemodynamics, fetal growth, and placental growth beginning on day 83 of gestation and continuing weekly until day 126 of gestation (Carr et al., 2012). By day 83 of gestation (baseline ultrasound measurements), the overnourished ewes already had decreased placentome size, and the placentomes remained smaller than those in control ewes for the remainder of the study. Placentome size in both the control and the overnourished ewes decreased throughout gestation (Carr et al., 2012). Fetal measurements, including biparietal distance and abdominal girth were significantly reduced in the overfed ewes compared with the controls, and in both groups, fetal measurements increased as gestation advanced (Carr et al., 2012). Umbilical artery RI and PI

were increased in the overnourished ewes by day 83 of gestation, and while these indices decreased in both the controls and the overfed ewes as gestation progressed, they remained increased in the overfed ewes compared with the control ewes (Carr et al., 2012).

In another established model of IUGR (Lekatz et al., 2010a, 2010b; Meyer et al., 2010a, 2010b), Lemley et al. (2012) fed pregnant ewes either a control diet, formulated to meet requirements or a restricted diet, formulated to provided 60% of requirements from day 50 until day 130 of gestation. In this experiment, noninvasive Doppler ultrasonography measurements were obtained on day 50 and repeated every ten days until day 110 of gestation. Measurements obtained included placentome size, fetal biparietal distance, fetal abdominal girth, umbilical artery RI and PI, and umbilical artery blood flow. Placentome size peaked around day 80 of gestation and decreased slightly afterwards. Reductions in fetal abdominal girth in the restricted ewes were not observed until day 110 of gestation, and nutritional plane did not impact biparietal distance in Lemley et al. (2012), but abdominal and biparietal distance increased as gestation advanced in both the control and restricted ewes. The umbilical artery RI and PI peaked prior to day 80 of gestation and decreased as gestation progressed in both treatment groups. Umbilical blood flow was decreased by day 80 of gestation in the restricted ewes and remained lower than umbilical blood flow in control animals throughout gestation(Lemley et al., 2012).

In both Carr et al. (2012) and Lemley et al. (2012), placentome diameter peaked by day 80 of gestation and RI and PI also peaked by day 80 of gestation before decreasing throughout the remainder of gestation. After attainment of maximal size, ovine placentome enhance their vascular development (Borowicz et al., 2007). Perhaps this increase in vascularity allows for the decrease in resistance indices as gestation advances (Newnham, et al., 1987; Wallace, et al., 2001; Acharya et al., 2004). Because differences in PI and RI are observed before differences in fetal size (Carr et al., 2012; Lemley et al., 2012) using these Doppler-derived indices may serve as an easy and noninvasive method to recognize compromised pregnancies earlier in gestation.

In conclusion, maternal nutritional plane appears to impact umbilical artery indices in sheep. More studies are needed to determine how monitoring umbilical resistance could help improve fetal outcomes.

Statement of the problem

Developmental programming is of interest to reproductive physiologists since stimuli or impairments early in life can alter fetal growth and development as well as the function of physiologic systems later in life (Godfrey and Barker, 2000). Epidemiologic evidence has identified poor maternal nutrition during pregnancy as a cause of intrauterine growth restriction (IUGR; Barker et al., 1993), and IUGR is a concern for both the human population and the livestock industry (Bernstein et al., 2000; Marsal, 2002; Wu et al., 2006; Mandruzzato, et al., 2008). While IUGR is a significant problem for both humans and livestock species, the development of effective therapeutic options has not been possible because of the lack of knowledge about the mechanisms of IUGR.

Animal models of poor nutrition have linked IUGR to inadequate blood flow to the fetus (Chandler et al., 1985; Wallace et al., 2001; Carr et al., 2012; Lemley et al., 2012). Because of the importance of placental blood flow to the developing fetus, it is not surprising that inadequate blood flow across the placenta is involved with IUGR.

Most of the available animal studies investigating IUGR involve global under- or over-nutrition during pregnancy; therefore, the question if the observed effects of maternal nutrition on fetal growth and development are due to overall nutrient levels or to levels of a specific component in the diet arises. Evidence in rodent models suggests that maternal protein level during gestation plays an important role in the health of the offspring; however, this data cannot be directly applied to ruminants, and data regarding maternal protein intake in ruminants is largely lacking.

Because rodent data indicates that maternal protein level may alter vascular function, particularly endothelium-dependent vasoresponses, and because bradykinin, which is dependent on functional endothelium, appears to be important for mediating blood flow during pregnancy, it is necessary to examine the role of maternal protein intake on vascular function of the placenta, including vasoactive pathways involving bradykinin. Such studies, which are currently nonexistent in sheep, will provide novel insight into the role of maternal protein intake and IUGR in sheep. Furthermore, these studies may provide insight to possible therapeutics for compromised pregnancies.

The first study (Chapter 2) in this dissertation hypothesizes that maternal global under- or over-nutrition during gestation will result in altered umbilical hemodynamics. The next study (Chapter 3) hypothesizes that maternal protein restriction will result in reduced amino acid concentration in fetal circulation, and the possible reduction in circulating arginine will reduce blood flow to the fetus. Thereafter, in Chapter 4, we hypothesize that maternal protein restriction will impair placental vascular function, particularly in response to bradykinin. Finally, in Chapter 5, the hypothesis was that placental arterial vasodilation is mediated at least partly through the nitric oxide pathway, and that maternal protein level may impact the pathways mediating bradykinin-induced vasodilation in the placental arteries.

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CHAPTER 2. IMPACTS OF MATERNAL NUTRITIONAL PLANE ON UMBILICAL ARTERY HEMODYNAMICS AND FETAL AND PLACENTOME GROWTH IN SHEEP

Abstract

The present study aimed to examine the impact of maternal nutritional plane on umbilical hemodynamics. Ewes (n = 15) were assigned to 1 of 3 dietary treatments [control (**CON**; 100% of NRC requirements), restricted (**RES**; 60% of CON), or overfed (**OVR**; 140% of CON)] beginning on day 40 of gestation. Umbilical artery hemodynamics, fetal growth, and placentome growth were measured on days 40, 45, 52, 80, 94, and 108 of gestation by Doppler ultrasonography. The percentage change in umbilical artery pulsatility and resistance indices remained steady through day 80 of gestation, and then decreased ($P \leq 0.03$) by day 108 of gestation. Moreover, plane of nutrition affected ($P = 0.03$) the percentage change from day 40 in pulsatility index, with RES ewes having a greater ($P = 0.03$) change compared to CON (16.66 vs. -15.57 ± 7.54%) with OVR being intermediate (3.19 ± 7.54%). Fetal biparietal and abdominal diameters increased ($P = 0.01$) throughout gestation, and fetal heart rate decreased ($P = 0.01$) from day 52 to 108 of gestation. Placentome diameter increased ($P = 0.01$) through day 80 of gestation, was similar ($P = 0.64$) at day 80 and 94 of gestation, and then decreased ($P = 0.01$) by day 108 of gestation. Maternal plane of nutrition can impact umbilical resistance indices, and ultimately may impact blood flow to the fetus.

Keywords: Maternal nutrition, pulsatility index, resistance index, sheep, umbilical artery

Introduction

Adequate blood flow to the fetus is critical for normal growth and development during gestation. In normal pregnancies, uterine and umbilical blood flows increase exponentially throughout gestation (Reynolds et al., 1995); however, in models of intrauterine growth restriction (**IUGR**) caused by maternal undernutrition or overnutrition, blood flow to the fetus is reduced (Chandler et al., 1985; Leury et al., 1990; Wallace et al., 2001; Carr et al., 2012; Lemley et al., 2012).

The pulsatility index (**PI**) and resistance index (**RI**) are pulsed-wave Doppler measurements of downstream resistance in arteries. These indices normally decline as gestation progresses and correlate positively with measured umbilical vascular resistance and negatively with umbilical blood flow (Newnham et al., 1987; Wallace et al., 2001; Acharya et al., 2004). Few studies have looked at umbilical artery PI and RI in sheep IUGR models, but Galan et al. (1998; 2005) and Carr et al. (2012) concluded that these indices are increased in the umbilical artery of compromised pregnancies.

We have demonstrated that first parity ewes fed 40% more, or 40% less, than adequately fed ewes, have lambs that are growth restricted at or near term (Lekatz et al., 2010a; Lekatz et al., 2010b; Meyer et al., 2010a, Lemley et al., 2012). We hypothesized the plane of nutrition will alter the umbilical artery PI and RI, which could have severe implications on blood flow to the fetus.

Materials and methods

The NDSU Animal Care and Use Committee approved all animal procedures for this study (#A0617). Nulliparous, Rambouillet ewes with similar (P = 0.73) body weight (52.6 ± 1.71 kg) carrying singleton fetuses (n = 15) were individually housed and on day 40 of gestation ewes were assigned randomly to 1 of 3 nutritional plane treatments supplying 60% (**RES**, n = 5), 100% (**CON**, n = 5) or 140% (**OVR**, n = 5) of global nutritional requirements (NRC, 1985; Meyer et al., 2010b). Ewes had free access to water and a trace mineralized salt block (Roto Salt Company, Penn Yan, NY). Diets were fed once daily at 0800 h in a complete pelleted ration. Ewes were weighed on day 40 of gestation and then every 7 or 14 days until day 130 at which time ewes were weighed for a

final ewe weight. Ewe body condition score was assigned (1 to 5 scale; 1 = emaciated, 5 = obese) by two trained technicians on days 40, 68, 96, 124, and 130, and the two body condition scores were averaged for each day. Diets were adjusted for body weight every 14 days.

Umbilical artery hemodynamics were assessed using a duplex B-mode (brightness mode; Figure 2.1) and D-mode (Doppler spectrum) program of the color Doppler ultrasound instrument (model SSD-3500; Aloka America, Wallingford, CT) fitted with a 7.5-MHz finger transducer (Aloka UST-672) as previously described (Lemley et al., 2012). Ultrasonography evaluations took place on days 40, 45, 52, 80, 94, and 108 of gestation. All dams were scanned transabdominally.

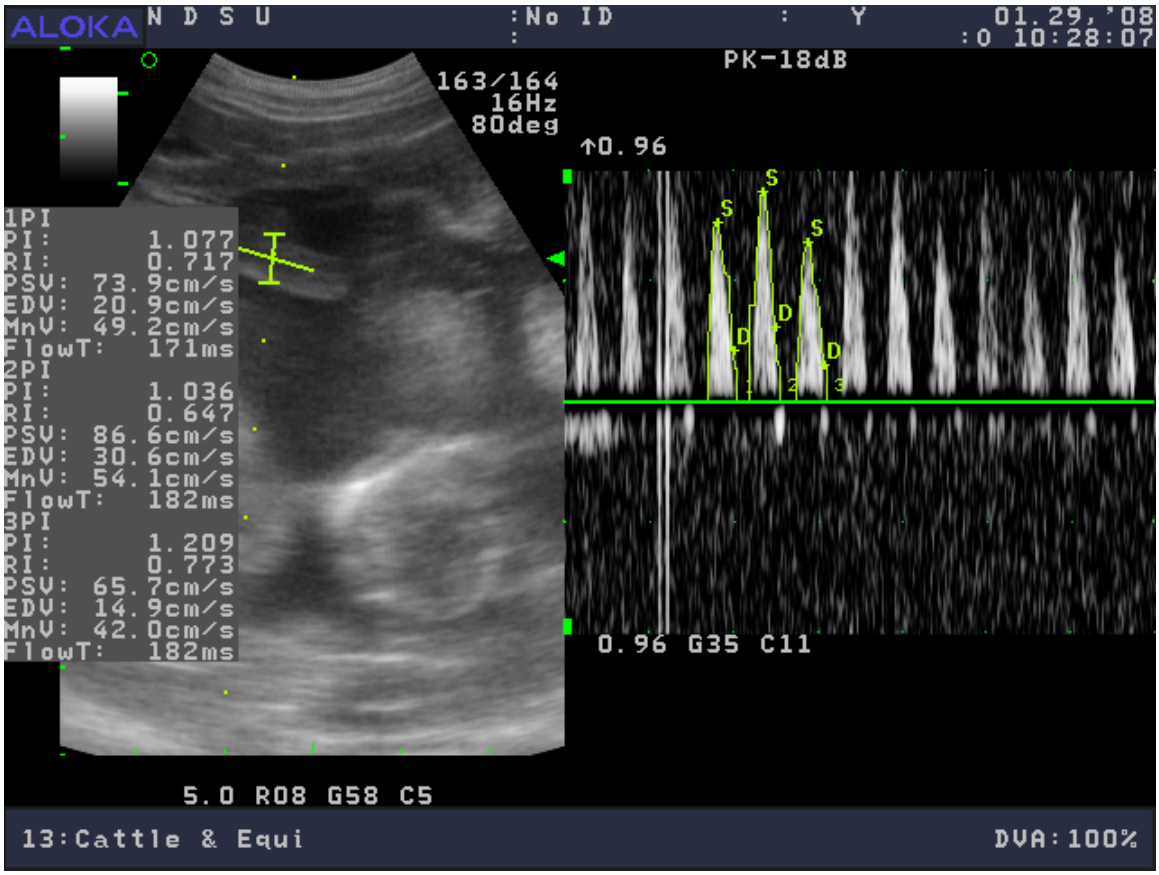

Figure 2.1. An image of umbilical cord hemodynamics with pulsatility index (**PI**) and resistance index (**RI**) measurements present on the left hand side. The umbilical cord was located, the angle of insonation was determined, and wave forms of the cardiac cycle were recorded. Measurements for at least 3 waveforms were averaged to determine PI and RI.

Briefly, for each ultrasound examination dams were placed into an elevated crate, wool from the abdomen and rear flanks was removed, skin cleaned with soapy water, and sufficient Aquasonic transmission gel (Parker Laboratories, Fairfield, NJ) was applied to the probe. Three similar cardiac cycle waveforms were obtained and averaged per ewe within a gestation day. Cardiac cycle waveforms were plotted in D-mode by velocity (cm/s; y-axis) and time (s; x-axis). Fetal heart rate (beats/min), pulsatility index (**PI**), and resistance index (**RI**) were calculated using preset functions on the instrument (Figure 2.1). Abbreviations for equations are: peak systolic velocity (**PSV**), end-diastolic velocity (**EDV**), and mean velocity (**MnV**). Equations are as follows: PI = [PSV (cm/s) – EDV (cm/s)] / MnV (cm/s); RI = [PSV (cm/s) – EDV (cm/s)] / PSV (cm/s). Percentage change in PI and RI were calculated as: [(value on any day – day 40 value) / day 40 value] × 100.

Placental and fetal growth parameters were assessed using B-mode. Fetal abdominal diameter was recorded at the base of the rib cage and above the entry point of the umbilicus (Figure 2.2). Fetal biparietal distance was also determined (Figure 2.3). Average placentome diameter was determined by randomly selecting ten placentomes and recording diameter at the largest position.

The effects of nutritional plane and day of gestation on ewe body weight, the percentage change from day 40 in ewe body weight, ewe body condition score the percentage change from day 40 in ewe body condition score, the percentage change from day 40 in umbilical artery PI and RI, fetal abdominal diameter, biparietal diameter, heart rate, and average placentome diameter were analyzed with the MIXED procedure of SAS (SAS software version 9.2; SAS Institute, Cary, NC) using repeated-measures ANOVA (d 40 to 108 of gestation), and if the F test was significant, means were separated using the PDIFF option of the LSMEANS statement. The model included nutritional plane, day of gestation, and their interaction. Least squares means and SEM are reported.

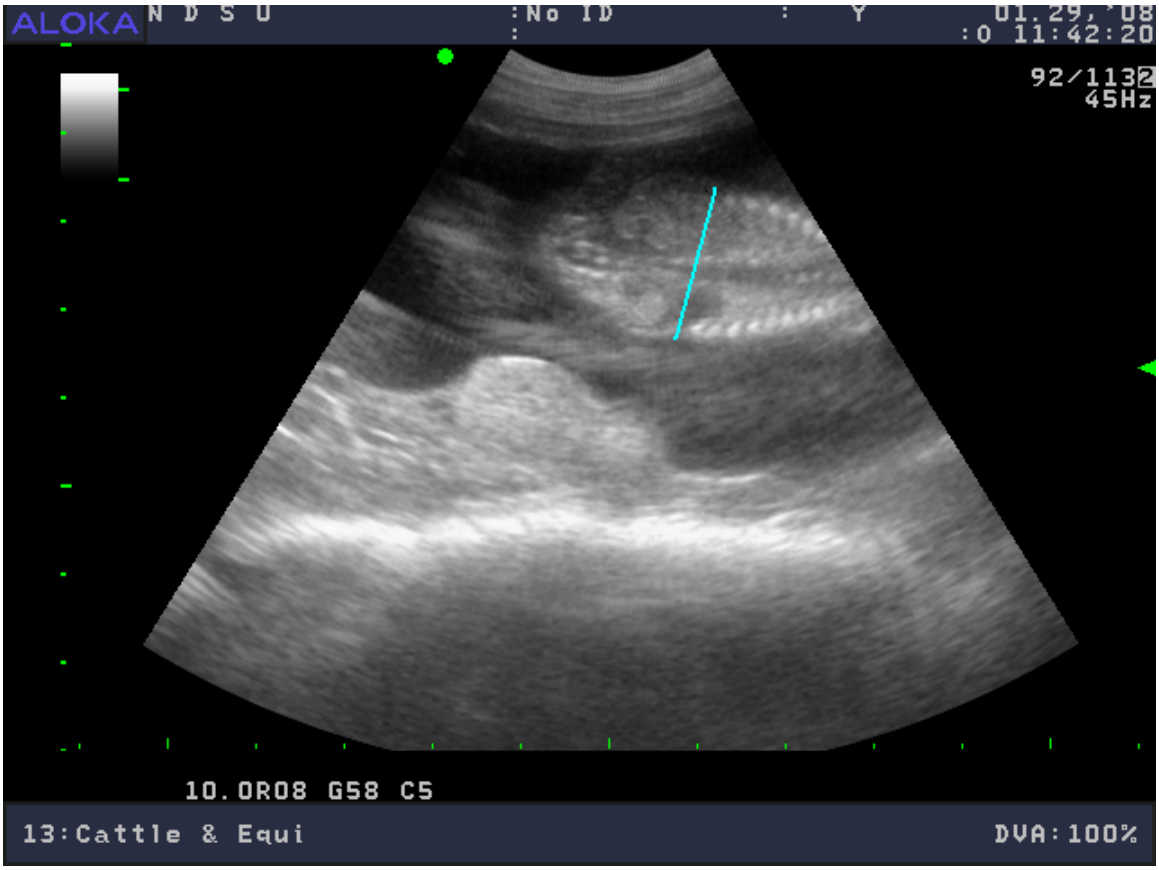

Figure 2.2. An image of a day 70 fetus. The blue line indicates the fetal abdominal width that was obtained by measuring across the body at the umbilicus. Left of the blue line, the outline of the kidney is apparent.

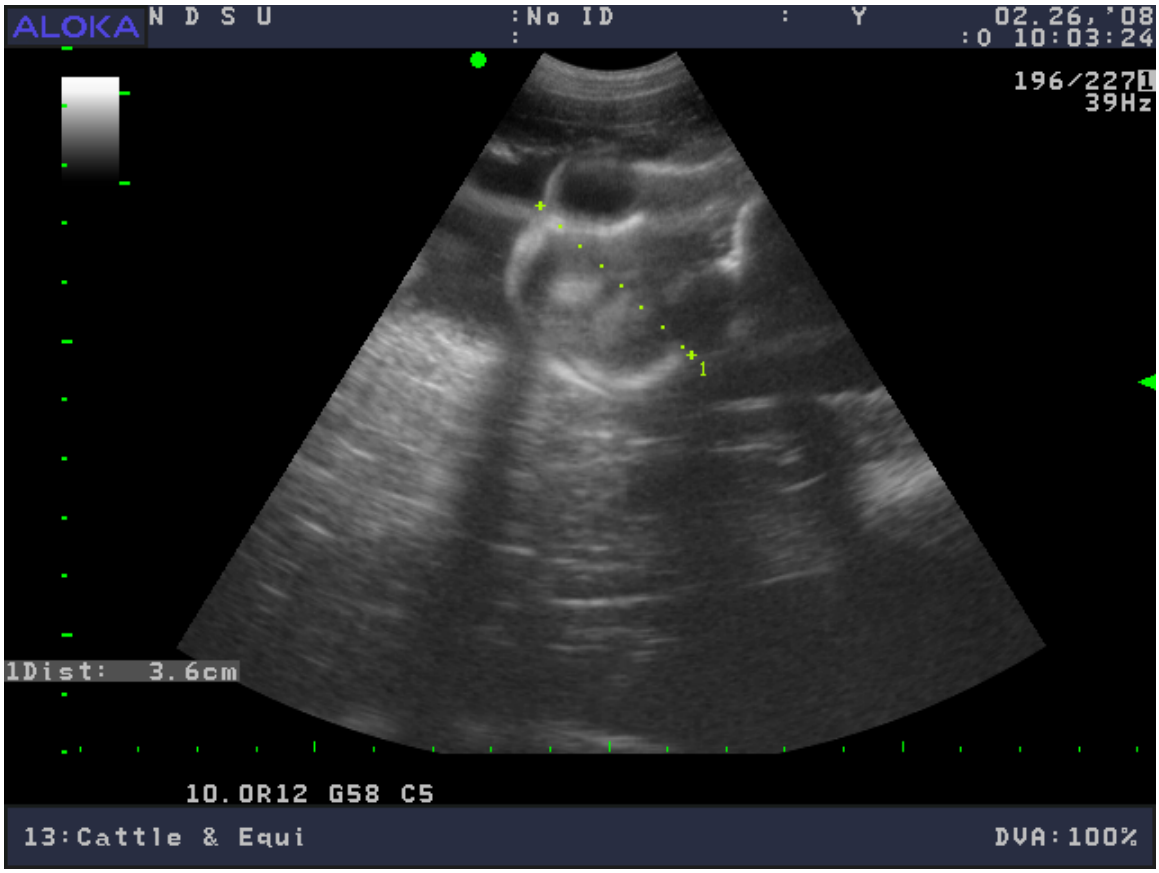

Figure 2.3. An image of a day 90 fetus where fetal biparietal diameter was obtained by measuring the width of the frontal bone immediately dorsal to the orbital sinus.

Results

There was a nutritional plane by day of gestation interaction (P = 0.01) for ewe body weight (Figure 2.4A), the percentage change from day 40 in ewe body weight (Figure 2.4B), ewe body condition score (Figure 2.4C), and the percentage change from day 40 in ewe body condition score (Figure 2.4D). Final ewe body weight was less (P = 0.01) in the RES ewes compared with the CON and OVR ewes, which did not differ (50.0 vs. 59.7 and 63.8 ± 2.81 kg for RES, CON, and OVR, respectively). The percentage change from day 40 to 130 in ewe body weight was different (P = 0.01) among all three groups (-3.66, 9.63, and 25.0 ± 2.16% for RES, CON, and OVR, respectively). Ewe body condition score on day 130 and the percentage change from day 40 top

130 of gestation in ewe body condition score were different among all three groups (body condition score: 2.4, 3.7, 4.3 ± 0.23 for RES, CON, and OVR, respectively and the percentage change in body condition score: -30.3, 4.66, 26.5 ± 5.33% for RES, CON, and OVR, respectively).

There was little evidence of a nutritional plane by day of gestation interaction for either the percentage change in umbilical artery PI (P = 0.60; Figure 2.5A) or RI (P = 0.72; Figure 2.5D). Both the percentage change in PI and RI were affected by day of gestation ($P \leq 0.03$; Figures 2.5B and 2.5E). Similar patterns were observed for percentage change in PI and RI with values remaining steady through day 80 of gestation where values numerically peaked, and decreasing thereafter (Figures 2.5B and 2.5E). Plane of nutrition also affected the percentage change in umbilical artery PI (P = 0.03; Figure 2.5C) where CON ewes decreased, and RES ewes increased (16.66 vs. -15.57 ± 7.54%) PI with OVR being intermediate (3.19 ± 7.54%). The effect of plane of nutrition on the percentage change in RI followed a similar pattern to that of percentage change in PI, but was not statistically different (P = 0.23; Figure 2.5F).

There was no plane of nutrition by day of gestation interaction ($P \geq 0.17$) or main effects ($P \geq 0.23$) for fetal biparietal diameter, abdominal diameter, heart rate measurements, or placentome diameter. As gestation advanced, fetal biparietal and abdominal diameters increased (P = 0.01) and fetal heart rate decreased (P = 0.01) from day 52 to 108 (Table 2.1). Placentome diameter increased (P = 0.01) through day 80 of gestation, was similar (P = 0.64) at day 80 and 94 of gestation, and then decreased (P = 0.01) by day 108 of gestation (Table 2.1).

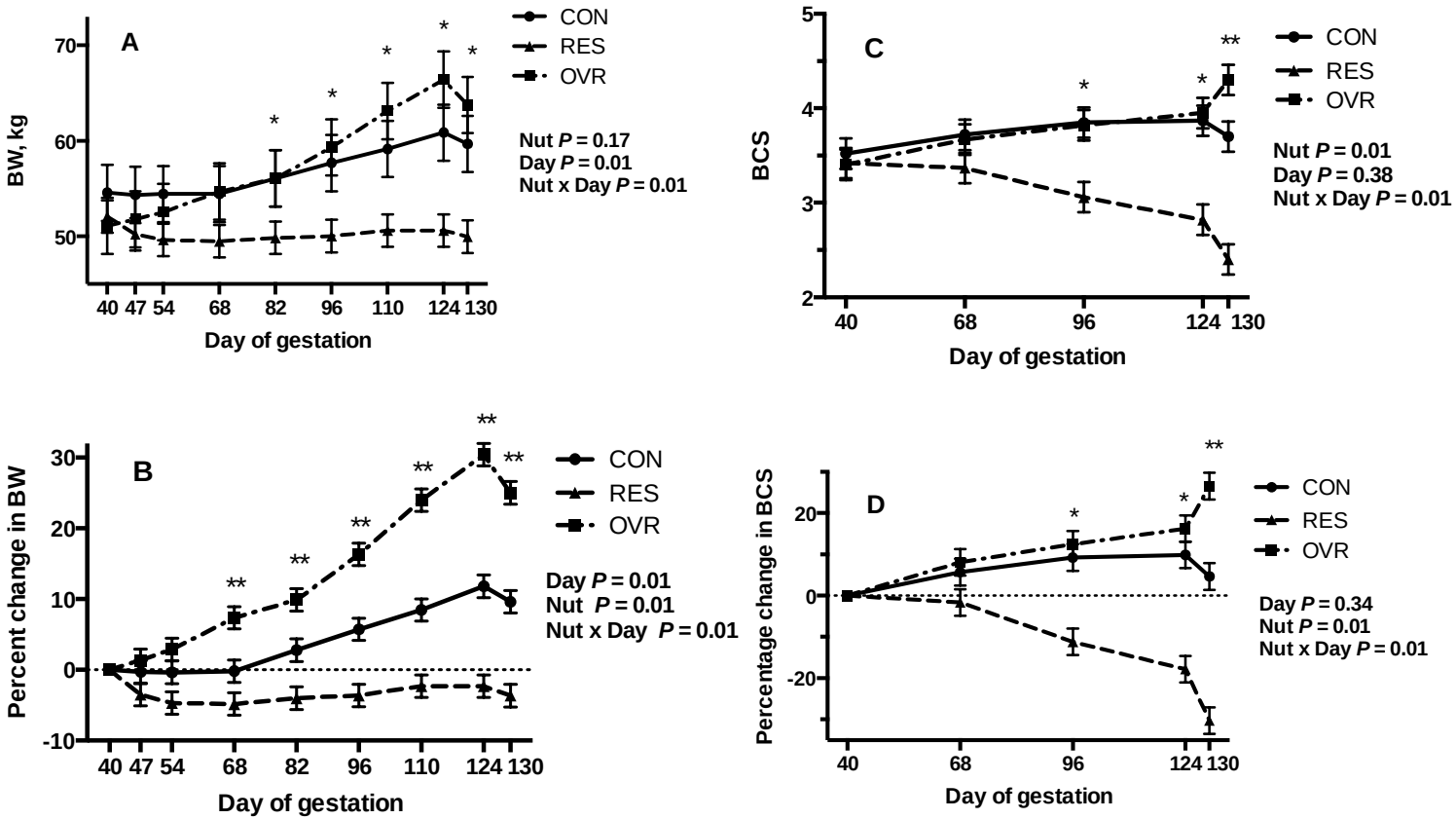

Figure 2.4. The interaction of maternal nutritional plane by day of gestation [Control (**CON**) = 100% of energy requirements; restricted (**RES**) = 60% of CON; overfed (**OVR**) = 140% of CON] on ewe body weight (**A, BW**), the percentage change from day 40 in ewe body weight (**B**), ewe body condition score (**C, BCS**), and the percentage change from day 140 in ewe body condition score (**D**). Percentage change in ewe body weight and ewe body condition score were calculated as [(value on any day - day 40 value) / day 40 value] × 100. * Means between RES and CON and between RES and OVR are statistically different ($P \leq 0.05$) on that particular day, with CON and OVR being similar ($P \geq 0.05$). **Means between RES and CON, RES and OVR, and CON and OVR are statistically different ($P \leq 0.05$) on that particular day.

Table 2.1. The interaction of nutrition and day of gestation and the main effect of day of gestation on fetal biparietal diameter, abdominal diameter, heart rate, and average placentome diameter[1] on days 40, 45, 52, 80, 94, and 108 of gestation.

	Measurement							
Day of gestation	Biparietal diameter, cm	SE	Abdominal diameter, cm	SE	Heart rate, beats per minute	SE	Average placentome diameter[1], cm	SE
P-value	0.01		0.01		0.01		0.01	
40	1.22[a]	0.03	1.27[a]	0.03	219[a]	1.06	1.12[a]	0.06
45	1.74[b]	0.04	1.74[b]	0.02	222[a]	2.62	1.73[b]	0.06
52	2.02[c]	0.03	2.37[c]	0.04	221[a]	1.89	2.17[c]	0.06
80	3.73[d]	0.09	4.58[d]	0.08	199[b]	3.14	2.70[d]	0.06
94	4.31[e]	0.11	6.71[e]	0.12	183[c]	3.00	2.68[d]	0.06
108	5.20[f]	0.11	7.33[f]	0.17	178[c]	2.63	2.42[e]	0.06

[1]Average placentome diameter was obtained by measuring the widest diameter of ten different placentomes and calculating the average.
[2]Biparietal diameter *P*-values: Nutrition by day interaction $P = 0.38$; Nutrition $P = 0.32$; Day $P = 0.01$
[3]Abdominal diameter *P*-values: Nutrition by day interaction $P = 0.17$; Nutrition $P = 0.23$; Day P = 0.01
[4]Heart rate *P*-values: Nutrition by day interaction $P = 0.17$; Nutrition $P = 0.63$; Day $P = 0.01$
[5]Average placentome diameter *P*-values: Nutrition by day interaction $P = 0.56$; Nutrition $P = 0.52$; Day $P = 0.01$
[abcdef]LSMeans with different superscripts differ by $P \leq 0.05$.

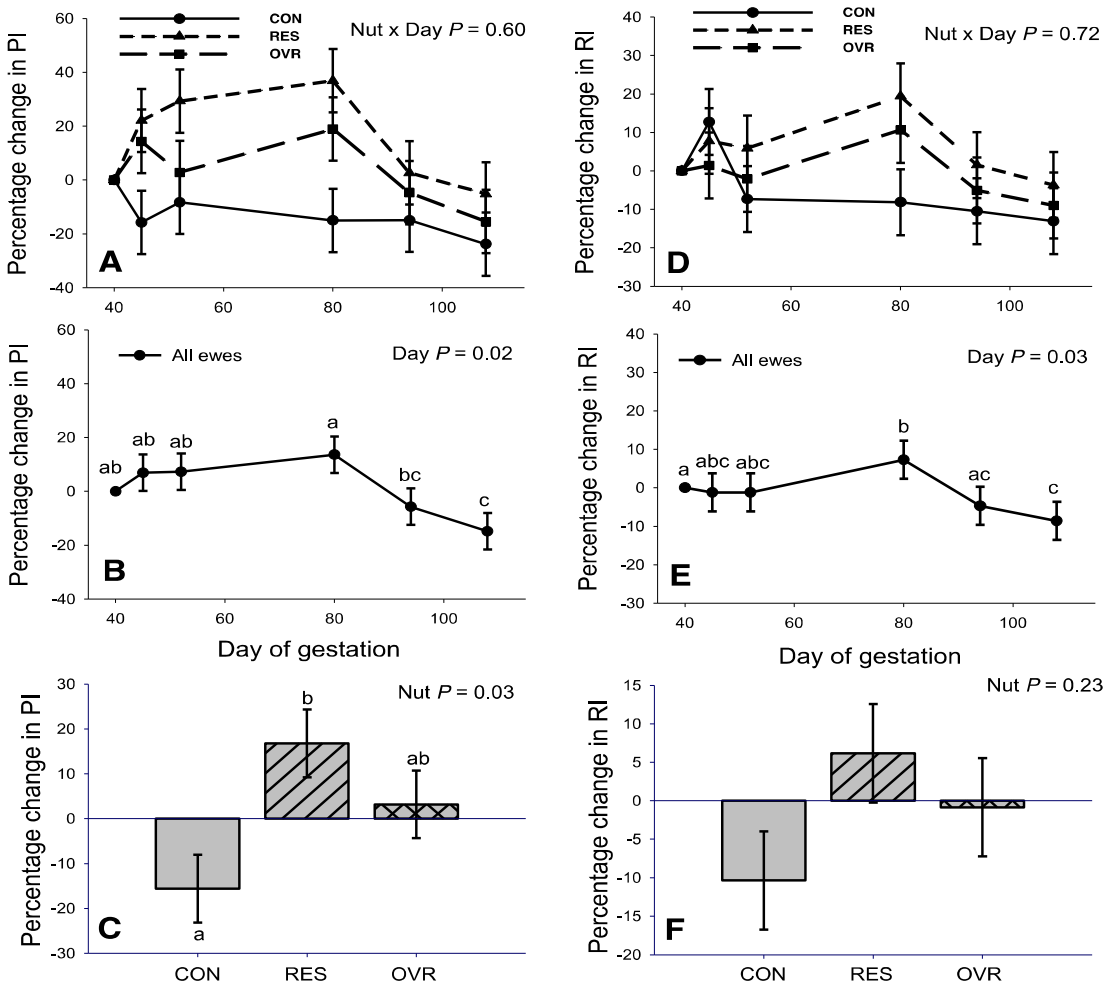

Figure 2.5. The impacts of maternal nutritional plane [Control (**CON**) = 100% of energy requirements; restricted (**RES**) = 60% of CON; overfed (**OVR**) = 140% of CON; C and F] and day (40, 45, 52, 80, 94, and 108 of gestation; B and E) and their interaction (A and D) on percentage change from day 40 in pulsatility index (**PI**; A, B and C) and resistance index (**RI**; D, E and F). Percentage change in PI and RI were calculated as [(value on any day – day 40 value) / day 40 value] × 100. Plane of nutrition dietary treatments were initiated on day 40. ^{abc}LSMeans with different subscripts differ by $P \leq 0.05$.

Discussion

Relatively few IUGR models in sheep have reported using Doppler ultrasonography to measure fetal and placental growth and umbilical artery indices throughout gestation. The limited data available, including the present study, are in agreement that fetal growth measurements increase with advancing gestation, placentome size peaks around d 80 of gestation, and umbilical artery PI and RI decrease throughout gestation in normal pregnancies (Galan et al., 1998; Galan et al., 2005; Carr et al., 2012; Lemley et al., 2012).

The ewes in this study were part of a larger study (Meyer et al., 2010a, 2010b) utilizing 84 ewes. The observed patterns for ewe body weight, the percentage change from day 40 in ewe body weight, ewe body condition score, and the percentage change from day 40 in ewe body condition score were expected due to the design of the study (Meyer et al., 2010b).

In the present study, fetal biparietal and abdominal diameters, measured via Doppler ultrasonography, increased with advancing gestation. This is in agreement with Carr et al. (2012) and Lemley et al. (2012) who also used Doppler ultrasonography to measure fetal growth throughout gestation. Using an established model of overfeeding adolescent ewes to result in IUGR, Carr et al. (2012) observed IUGR fetuses had smaller abdominal circumferences by day 98 of gestation and smaller biparietal diameters by day 110 of gestation compared to fetuses from control ewes, and these reductions in fetal growth remained through the end of ultrasound measurements at day 126 of gestation. When ewes were nutrient restricted beginning on day 50 of gestation, reductions in fetal abdominal girth were not observed until day 110 of gestation (Lemley et al., 2012). In the present study, maternal plane of nutrition did not alter fetal biparietal and abdominal diameters, however, ultrasound measurements ended at day 108 of gestation. Because maternal nutrition did not influence fetal growth measurements until later in gestation (Carr et al., 2012; Lemley et al., 2012), it is likely that differences in fetal growth measurements would have been observed in the present study if ultrasound measurements continued past day 108 of gestation.

Placentome diameter increased from day 40 to 80, and then decreased by day 108 of gestation. Similar patterns of placental growth have been reported in studies measuring

placentome diameter with ultrasound technology (Doizé et al., 1997; Carr et al., 2012; Lemley et al., 2012). The percentage change in PI and RI also peaked at day 80 gestation before decreasing through day 108 of gestation. This is similar to Lemley et al. (2012), where RI peaked at the time when placentome size peaked. After attainment of maximal size, ovine placentome enhance their vascular development (Borowicz et al., 2007). Perhaps this increase in vascularity allows for the decrease in resistance indices as gestation advances (Newnham, et al., 1987; Wallace, et al., 2001; Acharya et al., 2004).

Resistance indices (i.e. PI and RI) decreased ~10-15% in CON ewes, whereas a similar increase was observed in RES ewes. Because Doppler-derived measurements of PI and RI correlate negatively to blood flow (Acharya et al., 2004) these results could indicate reduced umbilical blood flow (Lemley et al., 2012) or reduced placentome vascularity (Luther et al., 2007) in the RES ewes compared with CON. Because differences in PI and RI are observed before differences in fetal size (Carr et al., 2012; Lemley et al., 2012) using these Doppler-derived indices may serve as an easy and noninvasive method to recognize compromised pregnancies earlier in gestation.

In conclusion, maternal nutritional plane appears to impact resistance indices of the umbilical artery in sheep. More studies are needed to determine how monitoring umbilical resistance could help improve fetal outcomes.

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CHAPTER 3. MATERNAL METABOLIZABLE PROTEIN RESTRICTION DURING LATE GESTATION ON UTERINE AND UMBILICAL BLOOD FLOW, FETAL ORGAN WEIGHTS, AND MATERNAL AND FETAL AMINO ACID CONCENTRATIONS NEAR TERM IN SHEEP

Abstract

To examine the effects of maternal metabolizable protein **(MP)** restriction during late gestation on uterine and umbilical blood flow, fetal weight, fetal organ weight, and amino acid concentrations in the uterine and umbilical vessels, a subset of 11 ewes with singleton pregnancies were assigned to one of three isocaloric diets that were formulated to provide 60% of MP **(MP60)**, 80% of MP **(MP80)**, or 100% of MP **(MP100)** requirements from day 90 to 130 of gestation. On day 130 of gestation, intraoperative uterine and umbilical blood flows were obtained as well as serum samples from the uterine artery, uterine vein, umbilical artery, and umbilical vein. Fetuses and placentas were collected and weighed, and fetal organs were harvested and weighed. Serum samples were analyzed for amino acid concentrations. Ewes on the MP60 diet had lighter ($P = 0.04$) fetuses, shorter fetal CCR ($P = 0.05$) and smaller fetal girth ($P = 0.03$) but increased ($P = 0.02$) uterine blood flow relative to fetal weight compared to the MP100 ewes, with MP80 being intermediate. Placental weight did not differ ($P = 0.41$) among treatments. Fetal adrenal, kidney, and liver weight was lighter ($P \leq 0.05$) in the MP60 ewes compared with the MP100 ewes, with MP80 ewes being intermediate and fetal lung and pancreas weight was lighter ($P \leq 0.02$) in the MP60 ewes compared to both the MP80 and MP100 ewes. However, when expressed relative to fetal weight, there were no organ weight differences ($P \geq 0.08$). Glutamine, glycine, leucine, ornithine, serine, and valine concentrations were all impacted ($P \leq 0.02$) by maternal metabolizable protein level. In summary, the MP60 diet resulted in lighter and smaller fetuses compared to the MP100 ewes despite the increase in uterine blood flow relative to fetal weight and similar placental sizes. Further, the MP60 diet resulted in lighter fetal organ weights. When the ratio of fetal organ weights and fetal weight was calculated, there were no treatment differences, indicating synchronous intrauterine growth restriction. This study

provides novel information regarding the effect of maternal metabolizable protein restriction on fetal growth, but further, and larger, studies are needed.

Key words: ewes, blood flow, late gestation, metabolizable protein

Introduction

Many models of intrauterine growth restriction caused by alterations to maternal diet in sheep exist (Chandler et al., 1985; Wallace et al., 2001; Carr et al., 2012; Lemley et al., 2012). Maternal nutrient restriction in sheep alters umbilical blood flow throughout gestation and uterine blood flow at day 130 of gestation (Lemley et al., 2012) and also decreases the concentrations of amino acids in the uterine artery and umbilical vein (Kwon et al., 2004; Satterfield et al., 2010). The question remains if these alterations to blood flow and amino acid concentrations are due to an overall global restriction of nutrients or if there is a specific nutrient or nutrients that is driving these changes. Evidence from rodent models of maternal protein restriction demonstrates reduced offspring birth weight, elevated blood pressure in the offspring, and decreased vascular responses of the uterine artery (Langley et al, 1994; Langley-Evans and Jackson, 1996; Itoh et al., 2002). While much has been learned about the effects of maternal protein restriction in rodents, these findings cannot directly be applied to a ruminant, and data regarding the effects of protein restriction on uteroplacental function during gestation in ruminants is largely lacking.

Therefore, for the purposes of this study, isocaloric diets with varied amounts of metabolizable protein were evaluated. Metabolizable protein (**MP**) is defined by the NRC (2007) as the true protein, which is derived from dietary and microbial protein that is digested post-ruminally and from which the constituent AA are absorbed from the intestine. Therefore, providing adequate MP during gestation may serve as a more appropriate indicator of how protein intake affects ruminant dams and their offspring.

We hypothesized that isocaloric diets provided to dams with lower amounts of MP would decrease fetal growth by reducing uteroplacental blood flow and nutrient delivery to the conceptus. Further, we hypothesized that MP restriction would reduce amino acid concentrations in uterine and umbilical vessels. Therefore, the objectives of this study were to evaluate the

effects of isocaloric diets with varied levels of MP during late gestation (day 100 to 130) on uterine and umbilical blood flow, fetal weight, fetal organ weight, and amino acid concentrations in the uterine and umbilical vessels at day 130 of gestation.

Materials and methods

Animal care and use were according to protocols approved by the North Dakota State University (**NDSU**) Animal Care and Use Committee (#A0921).

Animals and experimental design

The breeding protocol used for this study was previously published (Van Emon, 2013). On ~ day 90 of gestation, 45 pregnant multiparous ewes were transported from the Hettinger Research Extension Center (Hettinger, ND) to the Animal Nutrition and Physiology Center at NDSU (Fargo, ND) where they were housed in individual pens (0.91 × 1.2 m) in an indoor facility until necropsy (130 ± 2 days of gestation). Within the facility, the temperature was held constant at 12ºC, and lighting was controlled automatically (12:12-h light-dark cycle with lights on at 07:00 and of at 19:00).

Ewes were acclimated to low-quality hay (Table 3.1) and the MP100 supplement (100% of the MP requirements, as determined by NRC, 2007; Table 2) for 10 days prior to starting dietary treatments. Ewes were weighed on two consecutive days (days 99 and 100 of gestation) prior to the initiation of treatments. On day 100 ± 2 (SD) of gestation ewes were randomly assigned to one of three isocaloric dietary treatments (Table 3.2): **MP60**: 60% of MP requirements, **MP80**: 80% of MP requirements, and **MP100**: 100% of the MP requirements on a DM basis during the last 4 weeks of gestation (NRC, 2007; Van Emon et al., 2013). Dietary treatments were fed from 100 to 130 ± 2 days of gestation. Metabolizable protein intake was determined by the average of the days 99 and 100 body weight and offered once daily at 0700 h. Ewes were given one hour to consume the supplement, which was always completely consumed, then low-quality forage (Table 3.1) was offered. There were no orts throughout the study. Body weights were determined every 7 days throughout the dietary treatment period, and the amount

of supplement and low-quality forage offered was adjusted for changes in body weight. All ewes had access to fresh water and trace mineralized salt (4,000 ppm Zn, 1,600 ppm Fe, 1.200 ppm Mn, 325 ppm Cu, 100 ppm I, 40 ppm Co; American Stockman, Overland Park, KS).

Table 3.1. Nutrient composition of fescue straw[1].

Item	
Diet, % DM	
DM, %	96.24
NEm, Mcal/kg	2.22
CP, % of DM	2.76
MP, % of DM	1.95
NDF, % of DM	80.17
ADF, % of DM	48.66
Ash, % of DM	6.00

[1]Ewes were fed fescue straw to limit MP intake

Table 3.2. Ingredient and nutrient composition of dietary supplements fed to ewes.

	Treatment[1]		
Item	MP60	MP80	MP100
Ingredient, % DM			
Corn	18.50	15.00	5.00
DDGS[2]	7.00	20.00	30.00
Soyhulls	9.50	—	—
Nutrient composition			
DM, %	95.51	95.89	95.90
NEm, Mcal/kg	2.00	2.22	2.14
CP, % of DM	13.45	20.53	25.03
MP, % of DM	8.41	13.01	16.31
NDF, % of DM	33.61	32.11	40.79
ADF, % of DM	15.71	8.33	11.61
Ash, % of DM	3.17	3.50	4.38

[1]Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60: 60% of MP requirements; MP80: 80% of MP requirements; and MP100: 100% of MP requirements.

[2]Dried distillers grains with solubles

Gestational day 130 intraoperative ultrasonography measurements and blood samples

On day 130 ± 2 days of gestation, a subset (n = 12) of the 45 ewes carrying singletons [MP60: n = 4; MP80: n = 4; MP100: n = 3 (of this subset, one MP100 ewe was carrying twins and eliminated from the analysis)] underwent surgery to obtain intraoperative ultrasonography measurements as previously described by Lemley et al. (2012). Dams were weighed and anesthetized with 1.2 ml of 50 mg/ml pentobarbital sodium per 20 kg of body weight. Anesthesia was maintained via a jugular catheter. A catheter was placed into the maternal saphenous artery and advanced to the iliac artery via the femoral artery for monitoring and recording blood pressure throughout the procedure. The uterus was exposed via midventral laparotomy, covered with warm surgical towels, and a liberal amount of physiological saline (37ºC) was applied to the uterus every 5 min. The gravid uterine artery was located and a transonic flow probe (6 mm; Transonic Systems Inc., Ithaca, NY, USA) was placed around the gravid uterine artery prior to the first bifurcation of the uterine artery. Blood flow, as measured by the flow probe (Transonic Systems), was recorded. Intraoperative measurements of umbilical artery hemodynamics were assessed using a duplex B-mode (brightness mode) and D-mode (Doppler spectrum) program of the color Doppler ultrasound instrument (model SSD-3500; Aloka UST-672). Umbilical artery hemodynamics were recorded by scanning the gravid uterine horn in B-mode until a clear image of the umbilical artery was located. Measurements were obtained by placing the cursor over the vessel in B-mode while simultaneously recording pulsatile waves in D-mode. The average angle of insonation for intraoperative umbilical artery blood flow was 62.5 ± 9.49 degrees (mean ± SEM). Three similar cardiac cycle waveforms were recorded and averaged per ewe. Cardiac cycle waveforms were plotted in D-mode by velocity (cm/s; y-axis) and time (s; x-axis). Blood flow (**BF**) was calculated using present functions on the ultrasound instrument. Abbreviations for the blood flow equation are as follows: mean velocity (**MnV**) and cross-sectional area of vessel (**CSA**). The blood flow equation is BF (ml/min) = MnV (cm/s) × CSA (cm^2) × 60 s.

Gestational day 130 necropsy procedures

Following intraoperative ultrasound measurements, the gravid uterine horn was cut and the umbilical cord was excised. Blood samples were taken simultaneously from the uterine vein (of the gravid horn; **UtV**) and the iliac artery (via a catheter through the saphenous artery; maternal artery, **MA**) and then simultaneously from the umbilical artery (**UmbA**) and the umbilical vein (**UmbV**). Blood (~ 60mL) was collected into three 10-mL nonheparinized tubes (Becton Dickinson Vacutainer Systems). Serum was obtained by centrifugation (1,500 × *g* for 30 min) and stored at −20ºC until further analysis.

The fetus was removed, weighed, and exsanguinated. Fetal curved crown rump length and abdominal girth were recorded. Fetal organs were harvested and weighed. The placenta was collected, and placentomes were counted, dissected, and total placentome weight recorded. The caruncular and cotyledonary tissues were separated and weighed.

Amino acid analysis

Serum amino acid (**AA**) profiles were determined using an Ultra Performance Liquid Chromatograph (**UPLC**). Two-hundred fifty microliters of serum was deproteinized with 250 µL of 10% sulfosalicylic acid to which 250 µM norvaline was added as an internal standard. This mixture was vortexed and centrifuged for 5 min at 16,000 x g. Twenty microliters of supernatant was added to 60 µL of borate buffer and sodium hydroxide solution as well as 20 µL of MassTrac Amino Acid Analysis derivating reagent. The samples were then capped, mixed and heated in a digestion block at 55 ˚C for 10 min. Samples were then injected into the UPLC. This method utilizes the MassTrac Amino Acid Analysis system for the full profile of AA in physiological fluids. Derivatization chemistry for physiological samples is a precolumn method and is based on a derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, which converts both primary and secondary AA to stable chromophores for UPLC detection.

Calculations

Average placentome weight was calculated by dividing total placentome weight by placentome number. Uterine and umbilical blood flow per fetal weight was calculated by dividing uterine and umbilical blood flow by fetal weight, respectively. Gravid uterine, fetal, and uteroplacental uptake of amino acids were calculated. The following abbreviations were used for amino acid uptake calculations: **[aa MA]** = amino acid concentration in the maternal artery (iliac artery via the saphenous cathether), **[aa UtV]** = amino acid concentration in the uterine vein (gravid horn), **[aa UmbV]** = amino acid concentration in umbilical vein, **[aa UmbA] =** amino acid concentration in umbilical artery, **Uterine BF** = uterine artery blood flow, and **Umbilical BF** = umbilical blood flow. Equations for amino acid uptakes are as follows: gravid uterine uptake = ([aa MA nmol/ml] – [aa UtV nmol/nl]) × UtBF ml/min; fetal uptake = ([aa UmbV nmol/ml] – [aa UmbA nmol/ml]) × UmbBF ml/min; uteroplacental uptake = gravid uterine uptake – fetal uptake.

Statistical analysis

Initial ewe body weight, final ewe body weight, placental measurements, blood flow measurements, amino acid concentrations, amino acid uptakes, fetal measurements, and fetal organ weights were analyzed using the ordinary least squares [GLM procedure of SAS (SAS Institute, Cary, NC] with treatment in the model statement. Ewe body weight throughout the study was analyzed with the generalized least squares [MIXED procedure of SAS (SAS Institute)] with treatment, day of gestation, and the interaction of treatment by day of gestation in the model. Means were separated using the PDIFF option of the LSMEANS statement. Least square means and SE are reported.

Results

Ewe body weight

Initial (average of day 99 and 100; 67.0 ± 4.33 kg) or final (day 130; 66.6 ± 5.42 kg) ewe body did not differ (P = 0.85) among treatments. There was no treatment × day of gestation

interaction ($P \geq 0.77$) for either ewe body weight or the percentage change in ewe body weight (Figure 3.1A and 3.1C); however, there was an overall day effect ($P = 0.01$) on ewe body weight and the percentage change in ewe body weight (Figure 1B and 1D).

Uterine and umbilical blood flows

Uterine and umbilical blood flows are presented in Table 3. Maternal MP level tended ($P = 0.08$) to influence uterine blood flow, with the MP60 ewes having greater uterine blood flow compared with MP100 ewes, with the MP80 ewes being intermediate (Table 3.3). When expressed as blood flow per fetal weight, MP60 ewes had greater ($P = 0.02$) uterine blood flow per fetal weight compared with the MP80 and MP100 ewes, which were similar (Table 3.3). There was no effect ($P \geq 0.14$; Table 3.3) of maternal treatment on umbilical blood flow or umbilical blood flow per fetal weight.

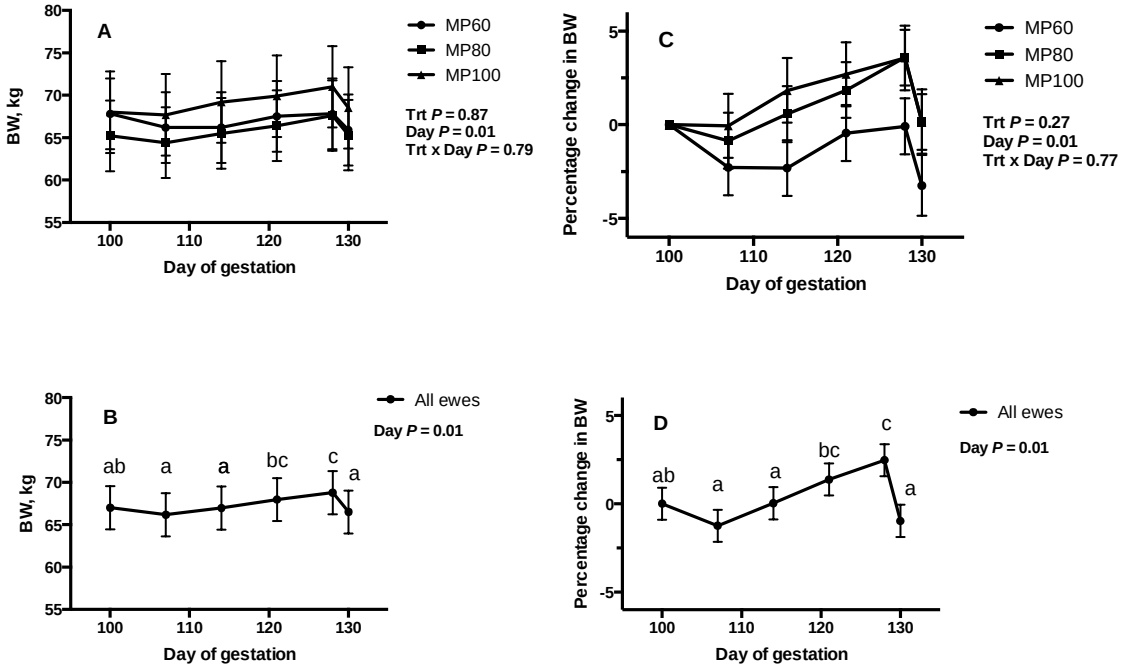

Figure 3.1. The effect of maternal metabolizable protein (**MP**) restriction on (**A**) ewe body weight throughout gestation and (**C**) the percentage change in ewe body weight throughout gestation and the effect of day of gestation on (**B**) ewe body weight and (**D**) the percentage change in ewe body weight throughout gestation. Maternal diets were formulated to provide 60% of MP requirements (**MP60**), 80% of MP requirements (**MP80**), or 100% of MP requirements (**MP100**). Diets were fed from day 100 to 130 of gestation. [abc]Means with different superscripts differ ($P \leq 0.05$).

Table 3.3. The effect of maternal metabolizable protein restriction from day 100 to 130 of gestation on uterine and umbilical blood flow measurements, conceptus weight, placentome weight and number, caruncular and cotyledonary weight, fetal membrane weight, and fetal weight, curved crown length, and girth measurements.

	Treatments[1]				
Measurement	60	80	100	SE	*P*-value
Uterine BF[2] (mL/min)	818	661	447	105	0.08
Uterine BF (mL/min) / fetal weight (g)	**0.42[a]**	**0.22[b]**	**0.12[b]**	**0.07**	**0.02**
Umbilical BF (mL/min)	387	425	328	90.9	0.70
Umbilical BF (mL/min) / fetal weight (g)	0.20	0.14	0.08	0.04	0.14
Conceptus weight[3] (g)	**2643[a]**	**3616[ab]**	**4330[b]**	**398**	**0.03**
Total placentome weight (g)	491	560	482	47.9	0.41
Total placentome number	63.0	75.2	54.7	15.4	0.61
Average placentome weight (g)	8.45	7.55	12.15	2.38	0.36
Total CAR[4] weight (g)	103	118	70.9	27.0	0.45
Total COT[5] weight (g)	331	386	352	43.3	0.60
Fetal membrane weight (g)	202	218	402	69.6	0.12
Fetal weight (g)	**2151[a]**	**3056[ab]**	**3848[b]**	**423**	**0.04**
Fetal CCL[6] (cm)	**50.8[a]**	**55.1[ab]**	**62.5[b]**	**3.02**	**0.05**
Fetal girth (g)	**28.2[a]**	**31.8[ab]**	**35.6[b]**	**1.61**	**0.03**

[1]Maternal diets (DM basis) were balanced for mature ewes baring twins during the last 4 weeks of gestation according to NRC (2007). Treatments: **MP60**: 60% of metabolizable protein (**MP**) requirements; **MP80**: 80% of MP requirements; and **MP100**: 100% of MP requirements. Diets were fed from day 100 to 130 of gestation.
[2]BF = blood flow
[3]Conceptus = placental weight plus fetal weight
[4]CAR = caruncular
[5]COT = cotyledonary
[6]CCL = curved crown length

Placental and fetal measurements

Placental and fetal measurement data are presented in Table 3. The MP60 ewes had a lighter (P = 0.03) conceptus weight compared with the MP100 ewes with the MP80 ewes being intermediate (Table 3.3). Total placentome weight, placentome number, average placentome weight, total caruncular weight, total cotyledonary weight, and fetal membrane weight did not differ ($P \geq 0.12$) among the treatment groups (Table 3.3). Fetuses from MP60 ewes were lighter (P = 0.04) compared with fetuses from MP100 ewes, with fetuses from MP80 ewes being intermediate (Table 3.3). Fetal CCR was shorter (P = 0.05) and fetal girth was less (P = 0.03) in MP60 ewes compared with fetal CCR in MP100 ewes, with MP80 ewes being intermediate (Table 3.3).

Fetal organ data is presented in Table 3.4. Fetal brain, large intestine, perirenal fat, small intestine, and thyroid weights did not differ ($P \geq 0.11$) among treatment groups (Table 3.4). Fetal heart, spleen, and stomach weights tended ($P \geq 0.06$ to $P \leq 0.09$) to be lighter in MP60 ewes compared with MP100 ewes (Table 3.4). Fetal adrenal glands, kidneys, and liver were lighter ($P \leq 0.05$) in fetuses from MP60 ewes compared with fetuses from MP100 ewes, with MP80 ewes being intermediate (Table 3.4). Fetal lung and pancreas weights were lighter ($P \leq 0.02$) in fetuses from MP60 ewes compared with fetuses from both MP80 and MP100 ewes, which were similar (Table 3.4). When evaluated on a organ weight per fetal weight (g/kg) basis, only fetal brain weight per fetal weight tended (P = 0.08) to differ among treatments with MP60 ewes having a heavier brain weight per fetal weight compared with the MP100 ewes, with MP80 ewes being intermediate (Table 3.4). All other organ weights per fetal weight did not differ ($P \geq 0.14$, Table 3.4).

Table 3.4. The effect of maternal metabolizable protein restriction from day 100 to 130 of gestation on fetal organ weights and fetal organ weight expressed relative to fetal weight.

	Treatments[1]				
Measurement	MP60	MP80	MP100	SE	*P*-value
Adrenal weight (g)	**0.22**[a]	**0.30**[ab]	**0.34**[b]	**0.03**	**0.05**
g / kg[2] fetal weight	0.10	0.10	0.09	0.01	0.51
Brain weight (g)	36.8	44.2	45.6	4.47	0.30
g / kg fetal weight	17.9	14.9	11.9	1.69	0.08
Heart weight (g)	15.4	21.1	26.0	2.98	0.07
g / kg fetal weight	7.12	7.02	6.76	0.30	0.66
Kidney weight (g)	**16.7**[a]	**20.3**[ab]	**25.6**[b]	**1.97**	**0.03**
g / kg fetal weight	8.00	6.87	6.63	0.52	0.15
Large intestine weight (g)	6.03	8.24	9.22	1.05	0.11
g / kg fetal weight	2.79	2.78	2.40	0.18	0.25
Liver weight (g)	**71.0**[a]	**78.7**[ab]	**102.0**[b]	**4.71**	**0.01**
g / kg fetal weight	34.5	28.1	26.5	4.42	0.37
Lung weight (g)	**79.8**[a]	**116**[b]	**139**[b]	**13.0**	**0.02**
g / kg fetal weight	38.5	38.5	36.2	2.47	0.73
Pancreas weight (g)	**2.08**[a]	**3.24**[b]	**3.88**[b]	**0.30**	**0.01**
g / kg fetal weight	1.03	1.09	1.01	0.11	0.86
Perirenal fat weight (g)	14.4	18.5	16.9	2.71	0.49
g / kg fetal weight	6.55	6.47	4.43	0.79	0.14
Small intestine weight (g)	20.3	32.5	30.4	8.66	0.51
g / kg fetal weight	9.10	10.63	8.07	1.99	0.63
Spleen weight (g)	4.32	5.77	5.34	0.47	0.09
g / kg fetal weight	2.10	2.17	1.39	0.47	0.44
Stomach weight (g)	15.7	21.7	27.3	3.01	0.06
g / kg fetal weight	7.29	7.25	7.08	0.45	0.94
Thyroid weight (g)	0.51	0.68	0.83	0.13	0.24
g / kg fetal weight	0.24	0.22	0.22	0.03	0.72

[1]Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments: **MP60**: 60% of metabolizable protein (**MP**) requirements; **MP80**: 80% of MP requirements; and **MP100**: 100% of MP requirements. Diets were fed from day 100 to 130 of gestation.
[2]Each organ weight is also expressed as ratio of organ weight (g) per fetal weight (kg)

Amino acids and uterine, fetal, and uteroplacental uptake

All amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery can be found in Supplement Table 3.1. Amino acids that differed ($P \leq 0.05$) among treatment groups are depicted in Table 3.5. Glutamine concentrations in the maternal artery, umbilical vein, and umbilical artery were not impacted ($P \geq 0.14$) by maternal protein level; however, glutamine concentrations in the uterine vein were greater ($P = 0.02$) in the MP60 and MP80 ewes compared with the MP100 ewes (Table 3.5). Glycine concentrations were also greater ($P \leq 0.04$) in the maternal artery, uterine vein, umbilical vein, and umbilical artery in the MP60 and MP80 ewes compared with the MP100 ewes (Table 3.5). Leucine concentrations in the maternal artery, uterine vein, and umbilical vein were not

different ($P \geq 0.06$) among the treatment groups, but leucine concentration in the umbilical artery were lower ($P = 0.04$) in the MP60 and MP80 ewes compared with the MP100 ewes (Table 3.5). Ornithine concentration in the maternal artery and uterine vein were greater ($P \leq 0.02$) in the MP80 ewes compared with both the MP60 and MP100 ewes (Table 3.5); however concentration in the umbilical artery and vein were not different ($P \geq 0.66$) due to treatment (Table 3.5). Serine concentration in the maternal artery, umbilical vein, and umbilical artery were not altered ($P \geq 0.08$) by maternal protein level (Table 3.5), but was greater ($P = 0.03$) in the uterine vein in MP60 and MP80 ewes compared with the MP100 ewes (Table 3.5). Valine concentration in the maternal artery and uterine vein were not different ($P \geq 0.39$) among the treatment groups (Table 3.5); however, in the umbilical vein, valine concentration was lower ($P = 0.04$) in the MP60 and MP80 ewes compared with the MP100 ewes, and in the umbilical vein, valine was lower ($P = 0.03$) in the MP60 ewes compared with the MP100 ewes, with MP80 ewes being intermediate (Table 3.5).

Discussion

There are many well-established models of intrauterine growth restriction in sheep, but until now, no studies exist examining the effects of maternal metabolizable protein during late gestation on uterine and umbilical blood flow, fetal organ weight, and amino acid concentrations in uterine and umbilical vessels. This novel study indicates that maternal metabolizable protein restriction from day 90 to 130 of gestation does not negatively impact ewe weight, or circulating amino acid concentrations. In fact, while maternal metabolizable protein restriction resulted in lighter fetuses, the restriction may also result in compensatory mechanisms, as indicated by the increase uterine blood flow per fetal weight and increase in certain amino acids. It should be stressed that this study, while the first of its kind, does have a small sample size (n = 11), and caution should be taken when interpreting the results. The study does provide preliminary data that may be useful when designing studies to further investigate the effects of maternal metabolizable protein restriction in sheep.

Table 3.5. The effect of maternal metabolizable protein restriction on select amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery.

	Treatments[1]				
Amino acid (nmol/mL)	MP60	MP80	MP100	SEM	*P*-value
Glutamine					
MA[2]	265	255	226	13.2	0.14
UtV[3]	**246**[a]	**229**[a]	**129**[b]	**11.4**	**0.02**
UmbV[4]	704	739	647	76.6	0.68
UmbA[5]	615	589	548	57.1	0.68
Glycine					
MA	**408**[a]	**369**[a]	**226**[b]	**46.1**	**0.04**
UtV	**417**[a]	**367**[a]	**178**[b]	**60.8**	**0.04**
UmbV	**648**[a]	**765**[a]	**429**[b]	**47.9**	**0.04**
UmbA	**595**[a]	**642**[a]	**388**[b]	**56.7**	**0.02**
Leucine					
MA	58.3	89.0	97.9	11.3	0.06
UtV	52.5	81.3	78.6	10.7	0.12
UmbV	126	160	244	31.0	0.06
UmbA	**101**[a]	**132**[a]	**219**[b]	**27.9**	**0.04**
Ornithine					
MA	**19.3**[a]	**35.0**[b]	**20.0**[a]	**2.60**	**0.01**
UtV	**16.9**[a]	**28.8**[b]	**13.3**[a]	**2.54**	**0.02**
UmbV	52.4	50.4	56.6	5.17	0.66
UmbA	59.7	49.5	54.6	4.98	0.70
Serine					
MA	45.2	42.9	33.0	3.58	0.08
UtV	**40.9**[a]	**38.0**[a]	**27.3**[b]	**2.97**	**0.03**
UmbV	640	742	503	98.0	0.24
UmbA	663	734	520	94.8	0.29
Valine					
MA	69.9	90.5	87.9	12.4	0.39
UtV	63.2	82.8	71.2	11.2	0.40
UmbV	**175**[a]	**235**[a]	**323**[b]	**36.4**	**0.04**
UmbA	**153**[a]	**214**[ab]	**302**[b]	**33.3**	**0.03**

[1]Maternal diets (DM basis) were balanced for mature ewes baring twins during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60: 60% of MP requirements; MP80: 80% of MP requirements; and MP100: 100% of MP requirements.
[2]MA = maternal artery (saphenous artery)
[3]UtV = uterine vein
[4]UmbV = umbilical vein
[5]UmbA = umbilical artery

In the present study, maternal dietary treatment did not alter ewe body from day 100 to day 130 of gestation. Van Emon et al. (2012) fed similar metabolizable protein supplements to a larger group of ewes (n = 295) from day 90 until lambing and found that ewes receiving less than the NRC recommendations for metabolizable protein (60% MP) weighed less than ewes meeting MP recommendations (100% MP) on days 114 and 142 of gestation. In the present study, a much smaller number of ewes were utilized (n = 11); perhaps statistical differences among treatments would have been observed with a larger sample size. Further, Van Emon et al. (2012) fed the dietary treatments from day 100 of gestation until lambing, whereas ewes were sacrificed on day 130 of gestation in the present study. It is possible that had the diets in the present study been fed until lambing, statistical differences in ewe body weight would have been observed. The sharp drop in ewe body weight and the percent change in ewe body weight between days 128 and 130 of gestation is likely due to the fact that all ewes in this study underwent surgery on day 130 of gestation, and were removed from food for 24 hours prior to surgery.

In the present study, ewes receiving 60% of MP requirements had increased uterine blood flow per fetal weight (ml/min/fetal body weight) but similar placental weights compared with the ewes meeting MP requirements. Despite the greater uterine blood flow per fetal weight, fetal weight was lighter, fetal CCR was shorter, and fetal girth was less in the MP60 ewes compared with the MP100 ewes. The diets in the current study were formulated to be isolcaloric, so even though ewes had similar caloric intake, fetal weight, CCR, and girth were all negatively altered by day 130 of gestation, which indicates that maternal metabolizable protein may play a key role in the growth of the developing fetus. The increase in uterine blood flow per fetal weight without an increase in placental weight could indicate a compensatory mechanism in the MP60 ewes.

Currently, no data exist on the effects of maternal metabolizable protein during late gestation on uterine and umbilical blood flow in sheep. Lemley et al. (2012) recorded intraoperative uterine and umbilical blood flow measurements following a similar procedure to the current study using a color-Doppler ultrasound. In this study, ewes received a control diet (100% of NRC requirements) or a globally-restricted diet (60% of NRC recommendations) from day 50 to day 130 of gestation. At day 130 of gestation, intraoperative ultrasound measurements showed a decrease in uterine blood flow in the

restricted group; however, when expressed as uterine blood flow per fetal weight, there were no differences between the two treatment groups (Lemley et al., 2012). Umbilical blood flow and umbilical blood flow per fetal weight were not different at day 130 of gestation (Lemley et al., 2012), which is similar to the current study. Also similar to the current study, Lemley et al. (2012) observed lighter fetal weight in the restricted ewes compared with the control ewes, but similar placental weight between the two groups. It is important to remember that Lemley et al. (2012) were studying the effects of a control diet versus a globally restricted diet, whereas in the current study, diets were formulated to be isocaloric and only differ in the level of metabolizable protein. Taking this into account, it appears that while an overall nutrient restriction may lead to decreases in uterine blood flow (Lemley et al., 2012), specifically restricting metabolizable protein may result in greater uterine blood flow per fetal weight. The current study suggests the decrease in fetal weight observed by Lemley et al. (2012) may be specifically due to a decrease in maternal protein in the diet.

In a well-established model of intrauterine growth restriction where adolescent ewes are overnourished, Wallace et al. (2008) demonstrated that both fetal weight and uterine blood flow were decreased in the overfed adolescent ewes compared with controls on day 135 of gestation. This study was examining the effects of overnourishing adolescent dams (Wallace et al., 2008), where the current study was specifically looking at metabolizable protein levels in multiparous ewes These variables make it difficult to compare and contrast the results from Wallace et al. (2008) and the present study.

While there is a wealth of literature examining the effects of maternal undernutrition on fetal and/or offspring organ weight, few studies exist that look specifically at the effects of maternal metabolizable protein in ruminants on fetal and/or offspring organ weight. In the current study, maternal protein restriction (MP60) tended to decrease fetal heart, spleen, and stomach weights compared with the control group (MP100), deceased the fetal kidney and liver weight compared with the controls, and decreased fetal lung and pancreas weight compared with both a moderately restricted metabolizable protein group (MP80) and the control group. This indicates that maternal metabolizable protein restriction does result in intrauterine growth restriction. When expressed as fetal organ weight per fetal weight, only the fetal brain tended to be different, with the MP60 ewes tending to have a heavier brain weight per fetal weight ratio compared with the MP100 ewes. This indicates a synchronous growth restriction in the

MP60 ewes with the brain weight being spared. He et al. (2013) restricted maternal protein intake during late gestation in goats and found that at birth, protein restriction decreased birth weight and offspring weights of the thymus, heart, and small intestine. When kid weights were expressed relative to birth weight, the thymus and small intestine were lighter in the protein-restricted goats compared to the controls (He et al., 2013). The goats in the He et al. (2013) study received similar protein requirements as the ewes in the current study (a 40% reduction in protein requirements, or fed 60% of requirements).

In the uterine artery, the only amino acids that were influenced by maternal protein restriction were glycine and ornithine. Glycine concentration in the maternal artery was greater in the protein-restricted groups compared with the control ewes. Ornithine concentration in the maternal artery was greatest in the MP80 ewes compared with the MP60 and MP100 ewes. In the umbilical vein, the only amino acid influenced by maternal diet was glycine, with both the protein-restricted groups having greater glycine concentrations in the umbilical vein compared with the control ewes. The lack of treatment differences in amino acid concentrations in the uterine artery and umbilical artery indicate that maternal protein restriction does not greatly affect amino acid concentrations in the uterine artery and umbilical vein. This is in contrast to ewes that are globally restricted. For example, when ewes were globally restricted to 50% of NRC requirements from day 28 to 115 of gestation and blood samples obtained from the uterine artery and umbilical vein, the concentrations of most amino acids in the uterine artery and umbilical vein were less compared with ewes that were fed to meet requirements (Satterfield et al., 2010). Also, when ewes were fed 50% of NRC requirements from either day 28 to 78 of gestation or from day 28 to 135 of gestation, amino acid concentrations in the uterine artery and umbilical vein were decreased at days 78 and 135 of gestation (Kwon et al., 2004). It is difficult to compare the current study to those of Satterfield et al. (2010) and Kwon et al. (2004) for a few reasons. First, the dietary treatments are very different between the current study and these two studies. Satterfield et al. (2010) and Kwon et al. (2004) examined a 50% global nutrient restriction whereas the current study is investigating isocaloric diets that differ in metabolizable protein. Further, Satterfield et al. (2010) fed the dietary treatments much earlier in gestation and collected blood samples at slaughter on day 115 of gestation. Kwon et al. (2004) also began dietary restriction early in gestation (d 28) and collected samples at days 78 and 135 of gestation.

In the present study, dietary treatments were fed during late gestation (day 90 to 130 of gestation), and blood samples obtained under anesthesia at day 130 of gestation.

In the current study, the uterine, uteroplacental, and fetal uptakes of each amino acid were calculated (Table 3.6) using amino acid concentrations in the uterine artery and vein, umbilical artery and vein, and the uterine and umbilical blood flows. Amino acid uptakes did not differ with treatment (Table 3.6), which indicates that maternal protein restriction is not affecting amino acid availability to the fetus.

In conclusion, maternal metabolizable protein restriction from day 90 to 130 of gestation does not appear to negatively affect ewe body weight, uterine or umbilical blood flow, or amino acid concentrations in the uterine artery and umbilical vein. Fetal weight was decreased in the most severely protein-restricted ewes, which indicates that maternal metabolizable protein significantly impacts fetal growth. Interestingly, this decrease in fetal weight occurred despite an increase in uterine blood flow (expressed relative to fetal weight) and without changes in placental weight. Future studies are needed to investigate possible compensatory mechanisms, such as increased blood flow and altered amino acid pathways, that may occur in dams experiencing reduced metabolizable protein during late gestation.

Table 3.6. The effect of maternal metabolizable protein restriction on amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery and the uterine, fetal, and uteroplacental uptakes of amino acids.

	Treatments[1]				
Amino acid (nmol/mL) **Amino acid uptake** (nmol/min)	MP60	MP80	MP100	SEM	*P*-Value
Alanine					
MA[2]	179	178	151	23.1	0.63
UtV[3]	162	171	124	24.3	0.36
Uterine uptake[4]	13672	5560	12646	4835	0.38
UmbV[5]	499	562	449	39.8	0.15
UmbA[6]	447	519	415	36.9	0.14
Fetal uptake[7]	19771	17664	13474	10032	0.88
Uteroplacental uptake[8]	-6099	-12105	2215	7491	0.35
Arginine					
MA	73.7	89.6	77.6	9.62	0.42
UtV	87.9	82.9	92.9	8.99	0.58
Uterine uptake	1609	4430	2440	1586	0.38
UmbV	109	100	120	11.3	0.45
UmbA	90.3	71.0	102	15.2	0.34
Fetal uptake	7462	12469	8978	5804	0.69
Uteroplacental uptake	-5853	-8040	-5893	5792	0.92
Asparagine					
MA	23.2	27.2	25.6	2.22	0.37
UtV	22.5	24.4	22.5	1.69	0.62
Uterine uptake	429	1827	1382	619	0.23
UmbV	93.6	92.0	78.5	11.6	0.60
UmbA	73.7	72.7	67.8	7.09	0.80
Fetal uptake	7541	7524	4922	4660	0.88
Uteroplacental uptake	-7111	-5697	-3087	4882	0.80
Aspartic acid					
MA	8.23	8.04	8.66	0.87	0.86
UtV	9.10	10.6	9.53	1.46	0.69
Uterine uptake	-609	-1602	-390	497	0.17
UmbV	9.71	8.22	7.40	1.11	0.32
UmbA	10.2	9.17	8.27	0.84	0.29
Fetal uptake	-142	-516	-175	317	0.49
Uteroplacental uptake	-467	-1085	-99.5	578	0.38

Table 3.6. The effect of maternal metabolizable protein restriction on amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery and the uterine, fetal, and uteroplacental uptakes of amino acids (continued).

	Treatments[1]				
Amino acid (nmol/mL) **Amino acid uptake** (nmol/min)	MP60	MP80	MP100	SEM	*P*-Value
Citrulline					
MA	82.3	77.6	77.7	12.9	0.94
UtV	79.7	71.1	72.3	11.9	0.82
Uterine uptake	1640	4417	2692	1985	0.54
UmbV	121	114	136	15.6	0.56
UmbA	121	115	136	14.8	0.59
Fetal uptake	-465	69.1	469	1509	0.87
Uteroplacental uptake	2105	4348	2975	2443	0.67
Glutamine					
MA	265	255	226	13.2	0.14
UtV	**246**[a]	**229**[a]	**129**[b]	**11.4**	**0.02**
Uterine uptake	14580	17089	15948	4994	0.92
UmbV	704	739	647	76.6	0.68
UmbA	615	589	548	57.1	0.68
Fetal uptake	36045	60885	43225	27843	0.68
Uteroplacental uptake	-21465	-43796	-23811	28791	0.72
Glutamic acid					
MA	75.3	70.0	70.0	3.88	0.49
UtV	78.3	86.0	73.6	11.9	0.73
Uterine uptake	-2346	-8561	-1582	4425	0.43
UmbV	27.0	33.4	28.8	6.13	0.69
UmbA	54.6	53.0	44.6	8.91	0.68
Fetal uptake	-10535	-8400	-7377	4800	0.84
Uteroplacental uptake	8189	-161	7010	8634	0.62
Glycine					
MA	**408**[a]	**369**[a]	**226**[b]	**46.1**	**0.04**
UtV	**417**[a]	**367**[a]	**178**[b]	**60.8**	**0.04**
Uterine uptake	-7428	1578	16771	8023	0.13
UmbV	**648**[a]	**765**[a]	**429**[b]	**47.9**	**0.04**
UmbA	**595**[a]	**642**[a]	**388**[b]	**56.7**	**0.02**
Fetal uptake	21627	39609	17950	17675	0.52
Uteroplacental uptake	-29056	-38031	6459	21468	0.29

Table 3.6. The effect of maternal metabolizable protein restriction on amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery and the uterine, fetal, and uteroplacental uptakes of amino acids (continued).

	Treatments[1]				
Amino acid (nmol/mL) **Amino acid uptake** (nmol/min)	MP60	MP80	MP100	SEM	*P*-Value
Isoleucine					
MA	41.3	59.8	56.8	7.70	0.18
UtV	37.1	54.0	45.9	6.79	0.19
Uterine uptake	3279	3742	4873	1487	0.72
UmbV	59.9	81.1	126	17.7	0.06
UmbA	50.0a	69.0ab	113b	16.0	0.05
Fetal uptake	3828	5538	4690	3102	0.76
Uteroplacental uptake	-549	-1796	-2.14	3663	0.90
Leucine					
MA	58.3	89.0	97.9	11.3	0.06
UtV	52.5	81.3	78.6	10.7	0.12
Uterine uptake	4459	5101	8468	2247	0.41
UmbV	126	160	244	31.0	0.06
UmbA	**101[a]**	**132[a]**	**219[b]**	**27.9**	**0.04**
Fetal uptake	9653	11512	11961	6165	0.94
Uteroplacental uptake	-5194	-6410	-909	6896	0.81
Histidine					
MA	34.6	36.2	31.2	3.03	0.48
UtV	32.8	33.7	20.8	2.83	0.33
Uterine uptake	1253	1773	1492	600	0.78
UmbV	56.9	61.7	52.7	8.87	0.56
UmbA	56.2	53.5	50.8	5.00	0.73
Fetal uptake	3663	2935	1089	2994	0.78
Uteroplacental uptake	-2411	-1162	424	3198	0.77

Table 3.6. The effect of maternal metabolizable protein restriction on amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery and the uterine, fetal, and uteroplacental uptakes of amino acids (continued).

	Treatments[1]				
Amino acid (nmol/mL) **Amino acid uptake** (nmol/min)	MP60	MP80	MP100	SEM	*P*-Value
Lysine					
MA	86.8	102	98.8	12.0	0.59
UtV	83.4	99.8	94.2	11.0	0.50
Uterine uptake	3278	1111	1737	2327	0.75
UmbV	150	141	133	22.2	0.84
UmbA	128	126	115	20.8	0.90
Fetal uptake	8932	6620	7529	4947	0.90
Uteroplacental uptake	-5654	-5509	-4890	4831	0.99
Methionine					
MA	12.4	15.0	13.0	1.50	0.38
UtV	11.2	13.2	13.2	1.34	0.29
Uterine uptake	909	1215	1242	353	0.72
UmbV	56.8	54.8	55.8	5.58	0.96
UmbA	48.5	45.4	48.8	5.06	0.84
Fetal uptake	3100	3897	3231	2118	0.93
Uteroplacental uptake	-2191	-2681	-1666	2195	0.93
Ornithine					
MA	**19.3[a]**	**35.0[b]**	**20.0[a]**	**2.60**	**0.01**
UtV	**16.9[a]**	**28.8[b]**	**13.3[a]**	**2.54**	**0.02**
Uterine uptake	1895	1113	2545	618	0.27
UmbV	52.4	50.4	56.6	5.17	0.66
UmbA	59.7	49.5	54.6	4.98	0.70
Fetal uptake	971	399	952	712	0.70
Uteroplacental uptake	-2191	-2682	-1666	2195	0.49
Phenylalanine					
MA	28.0	32.5	34.4	3.22	0.33
UtV	26.3	30.7	27.9	3.01	0.51
Uterine uptake	1167	1211	2927	629	0.12
UmbV	115	110	155	22.8	0.33
UmbA	98.8	90.3	138	20.1	0.23
Fetal uptake	5931	7888	8087	4591	0.89
Uteroplacental uptake	-4794	-6676	-4344	4761	0.89

Table 3.6. The effect of maternal metabolizable protein restriction on amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery and the uterine, fetal, and uteroplacental uptakes of amino acids (continued).

	Treatments[1]				
Amino acid (nmol/mL) **Amino acid uptake** (nmol/min)	MP60	MP80	MP100	SEM	*P*-Value
Proline					
MA	54.1	52.9	47.0	6.20	0.78
UtV	50.4	51.9	44.9	6.76	0.73
Uterine uptake	733	822	968	955	0.98
UmbV	171	187	159	13.1	0.31
UmbA	155	171	147	11.1	0.30
Fetal uptake	6485	7309	4669	3655	0.84
Uteroplacental uptake	-5753	-6488	-3357	3239	0.74
Serine					
MA	45.2	42.9	33.0	3.58	0.08
UtV	**40.9**[a]	**38.0**[a]	**27.3**[b]	**2.97**	**0.03**
Uterine uptake	3096	3139	2625	851	0.89
UmbV	640	742	503	98.0	0.24
UmbA	663	734	520	94.8	0.29
Fetal uptake	-7675	4375	-6856	6326	0.19
Uteroplacental uptake	10772	-1236	10058	6251	0.19
Taurine					
MA	40.0	81.3	98.7	20.2	0.13
UtV	39.3	82.3	97.6	21.4	0.15
Uterine uptake	463	-314	973	1500	0.81
UmbV	95.6	166	154	60.6	0.62
UmbA	87.6	162	153	58.3	0.56
Fetal uptake	2650	1056	157	2603	0.71
Uteroplacental uptake	-2187	-1371	577	4035	0.86
Threonine					
MA	42.7	49.3	44.2	6.52	0.70
UtV	39.3	44.0	38.0	5.67	0.69
Uterine uptake	2634	3623	2916	1410	0.84
UmbV	251	235	221	21.6	0.59
UmbA	229	209	203	23.7	0.68
Fetal uptake	8049	10822	9307	5710	0.89
Uteroplacental uptake	-5415	-7199	-5486	6176	0.95

Table 3.6. The effect of maternal metabolizable protein restriction on amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery and the uterine, fetal, and uteroplacental uptakes of amino acids (continued).

	Treatments[1]				
Amino acid (nmol/mL) **Amino acid uptake** (nmol/min)	MP60	MP80	MP100	SEM	*P*-Value
Tryptophan					
MA	18.6	15.0	15.4	1.78	0.27
UtV	18.1	15.0	13.7	1.85	0.22
Uterine uptake	312	144	778	283	0.28
UmbV	59.5	45.5	59.4	7.14	0.26
UmbA	55.3	40.3	54.3	6.88	0.21
Fetal uptake	1339	2129	2598	1921	0.85
Uteroplacental uptake	-1027	-1984	-1708	1744	0.86
Tyrosine					
MA	26.8	19.6	30.6	3.24	0.08
UtV	24.7	16.6	25.5	3.46	0.14
Uterine uptake	1694	1744	2272	789	0.84
UmbV	106	66.4	132	22.1	0.13
UmbA	94.9	57.5	119	20.2	0.12
Fetal uptake	3760	3138	6132	4284	0.85
Uteroplacental uptake	-2066	-1394	-3379	4174	0.93
Valine					
MA	69.9	90.5	87.9	12.4	0.39
UtV	63.2	82.8	71.2	11.2	0.40
Uterine uptake	5071	5153	7380	2286	0.71
UmbV	175a	235a	323b	36.4	0.04
UmbA	**153[a]**	**214[ab]**	**302[b]**	**33.3**	**0.03**
Fetal uptake	7167	9487	10231	4646	0.83
Uteroplacental uptake	-2096	-4334	-608	5097	0.82

[1]Maternal diets (DM basis) were balanced for mature ewes baring twins during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60: 60% of MP requirements; MP80: 80% of MP requirements; and MP100: 100% of MP requirements.
[2]MA = maternal artery (saphenous artery)
[3]UtV = uterine vein
[4]Uterine uptake = ([MA amino acids, nmol/ml] – [UtV amino acids, nmol/nl]) × uterine blood flow, ml/min
[5]UmbV = umbilical vein
[6]UmbA = umbilical artery
[7]Fetal uptake = ([UmbV amino acids, nmol/ml] – [UmbA amino acids, nmol/ml]) × Umbilical blood flow, mn/min
[8]Uteroplacental uptake= gravid uterine uptake – fetal uptake

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CHAPTER 4. THE EFFECTS OF MATERNAL METABOLIZABLE PROTEIN RESTRICTION DURING GESTATION ON THE VASCULAR FUNCTION OF MATERNAL AND FETAL PLACENTAL ARTERIES IN SHEEP

Abstract

We hypothesized that a maternal diet formulated to provide low levels of protein would result in decreased sensitivity of the caruncular (**CAR**) and cotyledonary (**COT**) arteries compared to placenta arteries from ewes receiving an isocaloric diet that was formulated to meet metabolizable protein (MP) requirements; therefore, our objective was to determine how maternal MP level impacted the vascular responses to bradykinin (**BK**) of CAR and COT arteries of sheep. Pregnant ewes were fed one of three isocaloric dietary treatments that provided 60%, 80%, or 100% of the MP requirements (**MP60, MP80,** and **MP100**, respectively). Diets were fed from day 100 to 130 of gestation. On day 130, ewes were necropsied, fetal and placental measurements were obtained, and CAR and COT arteries were analyzed for vascular reactivity and mRNA expression of selected genes. Dose response curves to BK, sodium nitroprusside (**SNP**), potassium chloride (**KCl**), and phenylephrine (**PE**) in CAR and COT arteries were performed. Maternal MP did not alter ($P \geq 0.24$) any fetal or placental measurements. As MP decreased, the sensitivity to a low dose of KCl increased ($P = 0.05$) in the COT arteries. There was an overall treatment effect in the CAR and COT arteries for the BK dose response curve, where CAR arteries of MP80 ewes were more sensitive ($P = 0.05$) to BK compared with MP60 and MP100 ewes, and COT arteries of MP60 and MP80 ewes were more sensitive ($P = 0.01$) to BK compared with MP100 ewes. There were no treatment effects ($P \geq 0.09$) on the SNP or PE dose response curves in CAR or COT arteries. The mRNA expression of BK receptors 1 and 2, endothelial nitric oxide synthase, and soluble guanylate cyclase were not altered ($P \geq 0.38$) by MP level during gestation. Results from this study indicate that while maternal MP restriction may not negatively impact fetal or placental growth, MP restriction appears to alter placental vascular function, which could alter nutrient availability to the conceptus.

Key words: bradykinin, ewes, metabolizable protein, pregnancy, vascular function

Introduction

Several animal models have linked maternal undernutrition during gestation to fetal growth restriction (Holst et al., 1986; 1992; Bloomfield et al., 2000; Vonnahme et al., 2003; Scheaffer et al., 2004; Lekatz et al., 2010a; 2010b; Lemley et al., 2012), placental growth restriction (McCrabb et al., 1991; 1992a; 1992b; Heasman et al, 1998; McMullen et al., 2005), or both (Charlton and Johengen, 1985; Faichney and White, 1987). Increasing uterine blood flow during the last half of gestation is critical for oxygen and nutrient delivery to the exponentially growing fetus, and alterations to placental blood flow vasculature may compromise the growth of the developing fetus (Ford, 1995; Meschia, 1983; Redmer et al., 2004; Reynolds et al., 2005; Reynolds et al., 2006). In sheep, maternal nutrient restriction reduces uterine (Lemley et al., 2012) and umbilical blood flow (Lemley et al., 2012) and increases resistance of the umbilical artery (Lekatz, Chapter 2). However, it is not clear if these alterations in placental function are due to an overall caloric restriction or to restriction of a specific component in the diet.

Evidence in rodent models suggests that maternal dietary protein level plays an important role in the health of the offspring. For example, maternal restriction of dietary protein results in offspring with glucose intolerance (Dahri et al., 1991; Langley et al., 1994), insulin resistance (Dahri et al., 1991), elevated blood pressure (Langley and Jackson, 1994; Ozaki et al., 2001), and vascular dysfunction (Torrens et al., 2002; Brawley et al., 2003). While much has been learned about the role of maternal protein level and offspring health in rodents, this data cannot be directly applied to ruminants. Furthermore, these rodent studies utilized diets that were not isocaloric, so the question if placental adaptations are due to a decrease in total energy intake or to a decrease in protein still remains. We hypothesized that lower levels of maternal MP would have negative effects on the vascular function of placental arteries. Therefore, the objective of the current study is to investigate the effect of isocaloric diets with varied levels of metabolizable protein (**MP**) on the vascular function of placental arteries.

Materials and methods

Animal care and use were according to protocols approved by the North Dakota State University Animal Care and Use Committee (#A0921).

Animals and experimental design

On day ~ 90 of gestation, 18 pregnant multiparous ewes carrying singletons were transported from the Hettinger Research Extension Center (Hettinger, ND) to the Animal Nutrition and Physiology Center at North Dakota State University (Fargo, ND) where they were housed in individual pens (0.91 × 1.2 m) in an indoor facility until necropsy (130 ± 2 days of gestation). Within the facility, the temperature was held constant at 12ºC, and lighting was controlled automatically (12:12-h light-dark cycle with lights on at 07:00 and of at 19:00).

Ewes were acclimated to low-quality hay (Table 4.1) and the MP100 supplement (100% of the MP requirements, as determined by NRC, 2007; Table 4.2) for 10 days prior to starting dietary treatments. Ewes were weighed on two consecutive days (days 99 and 100 of gestation) prior to the initiation of treatments. On day 100 ± 2 (SD) of gestation ewes were randomly assigned to one of three isocaloric dietary treatments (Table 4.2): **MP60**: 60% of MP requirements, **MP80**: 80% of MP requirements, and **MP100**: 100% of the MP requirements on a DM basis during the last 4 weeks of gestation of pregnant ewes (NRC, 2007). Dietary treatments were fed from 100 to 130 ± 2 days of gestation. Metabolizable protein intake was determined by the average of the days 99 and 100 body weight and offered once daily at 0700 h. Ewes were given one hour to consume the supplement then low-quality forage (Table 4.1) was offered. Body weights were collected every 7 days throughout the dietary treatment period, and the amount of supplement and low-quality forage offered was adjusted for changes in body weight. All ewes had access to fresh water and trace mineralized salt (4,000 ppm Zn, 1,600 ppm Fe, 1.200 ppm Mn, 325 ppm Cu, 100 ppm I, 40 ppm Co; American Stockman, Overland Park, KS).

Table 4.1. Nutrient composition of fescue straw[1].

Item	
Diet, % DM	
DM, %	96.24
NEm, Mcal/kg	2.22
CP, % of DM	2.76
MP, % of DM	1.95
NDF, % of DM	80.17
ADF, % of DM	48.66
Ash, % of DM	6.00

[1]Ewes were fed fescue straw to limit MP intake

Table 4.2. Ingredient and nutrient composition of dietary supplements fed to ewes.

	Treatment[1]		
Item	MP60	MP80	MP100
Ingredient, % DM			
Corn	18.50	15.00	5.00
DDGS[2]	7.00	20.00	30.00
Soyhulls	9.50	—	—
Nutrient composition			
DM, %	95.51	95.89	95.90
NEm, Mcal/kg	2.00	2.22	2.14
CP, % of DM	13.45	20.53	25.03
MP, % of DM	8.41	13.01	16.31
NDF, % of DM	33.61	32.11	40.79
ADF, % of DM	15.71	8.33	11.61
Ash, % of DM	3.17	3.50	4.38

[1]Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments: **MP60**: 60% of MP requirements; **MP80**: 80% of MP requirements; and **MP100**: 100% of MP requirements.

[2]Dried distillers grains with solubles

Tissue collection and fetal and placental measurements

On day 130 ± 2 days of gestation, were weighed and euthanized by captive bolt (Supercash Mark 2, Accles and Shelvoke Ltd., Sutton Coldfield, West Midlands, UK) followed by exsanguination. The gravid uterus was immediately dissected cranial to the cervix and weighed. The uterus was opened along the antimesometrial side, the umbilical cord was ligated, and the

fetus was removed, weighed, and fetal curved crown rump and abdominal girth were recorded. Immediately after the fetus was removed, the terminal branches of fetal placental arteries (cotyledonary arteries; **COT** arteries) were dissected and either immersed in cold physiological salt solution for the organ bath chamber study (described below) or snap frozen in liquid nitrogen and stored at -80ºC for qPCR (described below). These COT arteries, terminating directly into the cotyledon, were typically secondary branches of the umbilical artery. Following removal of the fetal placental arteries, the gravid uterine artery was located and maternal placental arteries (caruncular arteries; **CAR** arteries) terminating at the caruncle were dissected and dissected and either immersed in cold physiological salt solution for the organ bath chamber study (described below) or snap frozen in liquid nitrogen and stored at -80ºC for qPCR (described below). These CAR arteries, terminating directly into the caruncle, were typically 4^{th} or 5^{th} branches of the gravid uterine artery. Because placentome size has been shown to influence vascular reactivity (Vonnahme et al., 2008) similarly sized placentomes were selected for each animal.

Next, the fetal fluid was collected and the volume recorded. Placentomes were counted, dissected, weighed, and the CAR and COT tissues were separated and weighed. The fetal membranes and empty uterine weight were also weighed.

Organ chamber studies

After transfer to the laboratory, the CAR and COT arteries were dissected free from surrounding placental tissue, cleaned of adherent fat and connective tissue, and cut into rings 4-5 mm in length. Four rings were prepared from both CAR and COT arteries for each ewe. The endothelium remained intact (**E+**) in half of the CAR arterial rings and half of the COT arterial rings, while the other half of the arterial rings were denuded of endothelium (**E-**) by gently rubbing the intimal surface with a fine forceps.

The following drugs were used: KCl, phenylephrine (**PE**), norepinephrine (**NE**), bradykinin (**BK**), and sodium nitroprusside (**SNP**; Sigma Chemical, St. Louis, MO), and 9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (**U46619**; Cayman Chemical, Ann Arbor, MI). Drug solutions were prepared daily, kept on ice, and protected from light until used. All drugs were dissolved initially in double-distilled water. Drug concentrations are reported as final molar concentrations in the organ chamber. The composition of

the physiological salt solution was (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 11.1 glucose.

Caruncular (n = 4 for each ewe; E+: n = 2; E-: n =2) and COT arterial rings (n = 4 for each ewe; E+: n = 2; E-: n =2) were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution, as previously described (Tunstall et al., 2011; Shukla et al., 2012). Oxygen levels (95% O_2/5% CO_2) and temperature (37˚C) were maintained throughout the experiment. Each ring was suspended by means of two fine stainless-steel wire clips passed through the lumen; one clip was anchored inside the organ chamber, the other connected to a force transducer (Model FT03, Grass Instrument Company, Quincy, MA, USA). Isometric tension was measured and recorded on a Grass polygraph. The tissues were stretched progressively to the optimal point of their length-tension relationship, using KCl (20mM) to generate a standard contractile response. After this procedure, the rings were allowed to equilibrate at their optimal length-tension for one hour prior to further exposure to any vasoactive substances. The presence or absence of intact endothelium was confirmed in each preparation by contracting the rings with NE (10^{-6} M) and observing the presence or absence of relaxation to the endothelium-dependent vasodilator, BK (10^{-7} M).

Concentration-response curves to KCl (10 to100 mM) and phenylephrine (10^{-8} M - 10^{-5} M) were obtained in both endothelium intact (E+) and endothelium-removed (E-) rings for both the CAR and COT arterial rings. The remaining CAR and COT arterial rings were contracted with U46619 (1 to 3 x 10^{-9} M), a thromboxane A_2-mimetic. After the U46619-induced contraction had reached a stable plateau, relaxation responses to increasing concentrations of the endothelium-dependent vasodilator BK1(10^{-9} to10^{-6} M) or the endothelium-independent vasodilator, SNP (10^{-9} to 10^{-6} M) were obtained. At the end of the concentration-response curves to the contractile agonists, arterial rings were stimulated with 60 mM KCl to determine the maximum tissue contraction.

Quantification of mRNA

The relative mRNA expression of endothelial nitric oxide synthase (**eNOS**), soluble guanylate cyclase (**GUCY1B3**), bradykinin receptor 1 (**BKR1**), and bradykinin receptor 2 (**BKR2**) was determined in the CAR and COT arteries. Caruncular and COT arteries (30 mg) were homogenized using a Polytron

homogenizer fitted with a 7 mm generator. Messenger RNA was isolated using a QIAshredder spin column and the RNeasy Mini Kit as described by the manufacturer (Qiagen, Valencia, CA). RNA was quantified by measuring the absorbance on a Nanodrop 2000c spectrophotometer. Synthesis of cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit as described by the manufacturer (Qiagen). This protocol includes a genomic DNA removal step, which was performed for all samples. Quantitative PCR (**qPCR**) reactions for eNOS and sGC contained the following: 1x TaqMan Universal PCR Master Mix (Life Technologies), forward and reverse primers (each 1 μM), water and cDNA for a final volume of 12.5 μl. Reactions to detect BKR1 and BKR2 contained the following: 1X SYBR Green PCR Master Mix (Life Technologies), forward and reverse primers (each 500 nM), water and cDNA for a final volume of 12.5 μl. Dissociation curves confirmed a single amplification species for both BKR1 and BKR2. Amplification of 18s rRNA using a 20X predesigned assay reagent, which contained both forward and reverse primers and a probe (Life Technologies), was performed for each sample and was used to normalize expression of each gene. The ratio of the gene of interest to 18S rRNA was used for quantifying the gene expression and is the data presented.

Data analysis

One MP100 ewe was removed from the study as she was carrying triplets. Contractile responses are expressed as a percentage of the maximal contraction to KCl (60 mM). Relaxation responses are expressed as a percentage of the initial tension induced by U46619. For each vasoactive agent, the concentration necessary to produce 50% of its own maximal response (**EC_{50}**) was determined using Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Briefly, the log[M concentration] was plotted along the x-axis and percent of maximal contraction plotted along the y-axis. Next, Prism 6 software was used to find the best fit curve using nonlinear regression with the dose response equation for log(dose) vs. response. The EC_{50} values were converted to the negative logarithms and expressed as -log molar EC_{50} (**pD_2**). Initial and final ewe body weight, fetal measurements, placental measurements [including the average placentome weight, calculated as: (total placentome weight / placentome number)], placental efficiency [calculated as: (total fetal weight per ewe / total placentome weight per ewe)], and the qPCR data, were analyzed by ordinary least squares [GLM procedure of SAS (SAS software version 9.2; SAS

Institute, Cary, NC)], with treatment in the model statement. The dose response curves for BK and SNP were also analyzed using ordinary least squares [GLM procedure of SAS (SAS Institute)], with treatment, dose, and the treatment by dose interaction in the model statement. The dose response curves for KCl and PE were also analyzed using ordinary least squares [GLM procedure of SAS (SAS Institute)], with treatment, dose, endothelium, the treatment by dose interaction, the treatment by endothelium interaction, the endothelium by dose interaction, and the treatment by endothelium by dose interaction in the model statement. The pD_2 values were analyzed using Prism 6 software (GraphPad Software, Inc.). Ewe body weight throughout gestation and the percentage change from day 100 in ewe body weight {calculated as: [(weight on any day – day 100 weight) / day 100 weight] × 100} were analyzed using generalized least squares [MIXED procedure of SAS (SAS Institute)] using repeated-measures ANOVA (day 100 to day 130 of gestation), with treatment, day of gestation, and the treatment by day of gestation interaction in the model. For all data, if the F-test was significant, means were separated using the least significant difference. Least squares means and SEM are reported.

Results

Ewe body weight and conceptus measurements

Initial ewe body weight did not differ (P = 0.11) among treatments (62.6, 73.1, and 68.7 ± 3.55 kg for MP60, MP80, and MP100, respectively). The MP60 ewes were lighter (P = 0.02) at slaughter compared with the MP80 ewes, with MP100 ewes being intermediate (62.8 vs. 79.1 ± 3.78 kg, and 71.6 ± 3.35 kg for MP60, MP80, and MP100 ewes, respectively). There was a treatment by day interaction for ewe body weight throughout gestation (Figure 4.1), with MP60 ewes being lighter (P = 0.03) than MP80 ewes on days 107, 114, 121, 128, and 130, with the MP100 ewes being intermediate (Figure 4.1A). There was also a treatment by day interaction for the percentage change from day 100 in ewe body weight (Figure 4.1), with MP60 ewes having a lower (P = 0.03) percentage change in body weight compared with MP100 ewes on day 107, with MP80 ewes being intermediate (Figure 4.1B).

Moreover MP60 ewes had a lower (P = 0.03) percentage change in body weight compared to both the MP80 and MP100 ewes on days 114, 121, 128, and 130 of gestation (Figure 4.1B). There was no statistical difference ($P \geq 0.24$) on any fetal or placental measurement observed among the maternal metabolizable protein levels (Table 4.3).

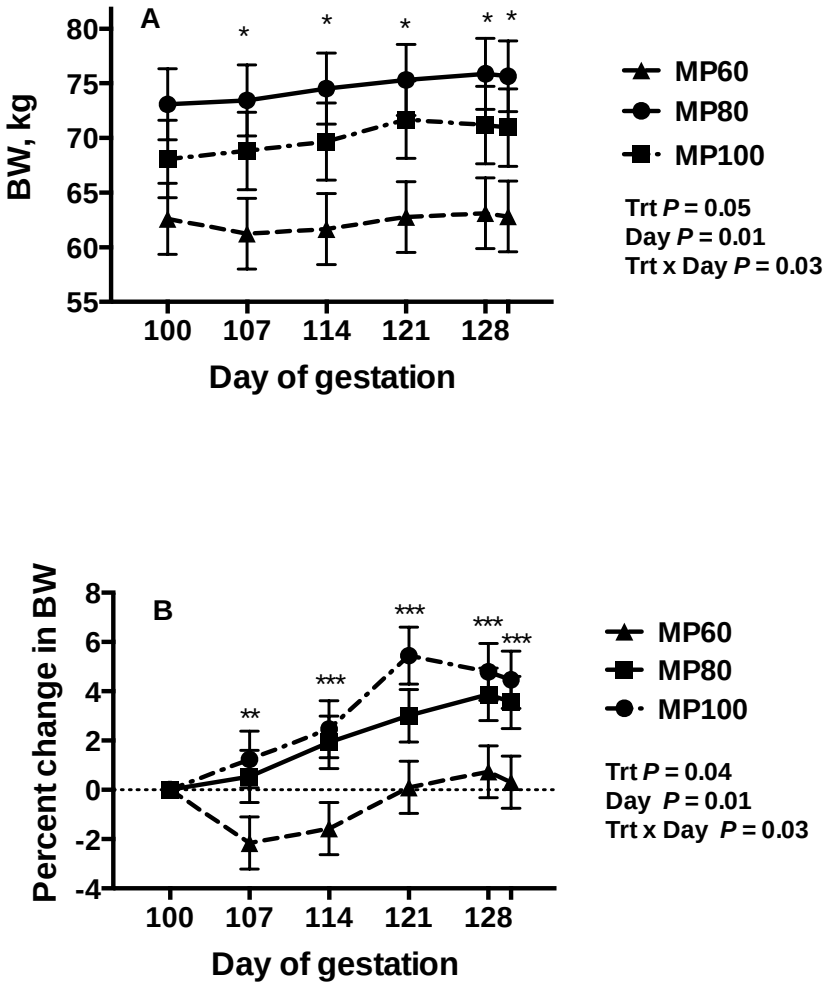

Figure 4.1. The effect of maternal metabolizable protein (**MP**) restriction on (**A**) ewe body weight throughout gestation and (**B**) the percentage change in ewe body weight throughout gestation. Maternal diets were formulated to provide 60% of MP requirements (**MP60**), 80% of MP requirements (**MP80**), or 100% of MP requirements (**MP100**). Diets were fed from day 100 to 130 of gestation. *MP60 differs from MP80 ($P \leq 0.05$), **MP60 differs from MP100 ($P \leq 0.05$), ***MP60 differs from MP80 and MP100 ($P \leq 0.05$) and MP80 and MP100 were similar ($P > 0.05$).

Table 4.3. The effect of maternal metabolizable protein level on gravid uterine weight, total fetal weight, average fetal weight, fetal curved crown length, fetal girth, total placentome weight, total placentome number, average placentome weight, total caruncular weight, total cotyledonary weight, fetal membrane weight, fetal fluid volume, empty uterine weight, and fetal weight to placental weight ratio.

	Treatments[1]				
Measurement	MP60	MP80	MP100	SE	*P*-value
Gravid uterine weight (g)	9277	9756	9348	1097	0.95
Total fetal weight[2] (g)	2450	2908	2602	445	0.80
Average fetal weight (g)	2110	2485	2187	443	0.79
Fetal CCL[3] (cm)	54.6	58.7	53.1	2.42	0.24
Fetal girth (g)	32.6	33.8	31.3	1.60	0.51
Total placentome weight[4] (g)	754.1	780.8	774.3	60.9	0.93
Total placentome number	80.0	89.5	79.7	7.10	0.50
Average placentome weight (g)	9.24	9.16	9.76	1.19	0.91
Total CAR[5] weight (g)	123.3	126.5	122.5	12.7	0.97
Total COT[6] weight (g)	573.7	595.9	592.4	47.7	0.92
Fetal membrane weight (g)	336.9	347.5	333.1	42.4	0.97
Fetal fluid volume (mL)	2212	2304	2299	298	0.97
Empty uterine weight (g)	66937	782.1	773.3	70.8	0.38
Fetal weight/total placentome weight (g/g)	4.10	4.04	3.78	0.83	0.94

[1]Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments: **MP60**: 60% of MP requirements; **MP80**: 80% of MP requirements; and **MP100**: 100% of MP requirements.
[2]Total fetal weight = total fetal weight per ewe
[3]Fetal CCL = fetal curved crown length
[4]Total placentome weight = total placentome weight per ewe
[5]CAR = caruncular
[6]COT = cotyledonary

Organ bath studies

Bradykinin (BK) dose response curves. The results of the BK dose response curve can visualized in Figures 4.1A and 4.1C for CAR and COT arteries, respectively. There was little evidence of a treatment by dose interaction on the BK dose response curve ($P \geq 0.45$) for either the CAR or the COT arteries (Figures 4.1A and 4.1C); however, there was an overall treatment effect ($P \leq 0.05$) on the BK dose response curve for both the CAR and COT arteries. In the CAR arteries, there is a slight leftward shift ($P = 0.01$; Figure 4.1A) in the MP80 ewes compared with the MP60 and MP100 ewes, which did not differ (pD_2: 7.87 vs. 7.66, 7.65 ± 0.06 for MP80, MP60, and MP100, respectively). In the COT arteries, there is a leftward shift of both ($P = 0.01$; Figure 4.1C) the MP60 and MP80 ewes compared with the MP100 ewes (pD_2: 8.07 vs. 7.90 vs. 7.36 ± 0.07 for MP60, MP80, and MP100, respectively).

Sodium nitroprusside (SNP) dose response curves. The results of the SNP dose response curve are visualized in Figures 4.1B and 4.1D for CAR and COT arteries, respectively. There was not a treatment by dose interaction ($P \geq 0.47$) for either the CAR or COT SNP dose response curve (Figures 4.1B and 4.1D). Further, there were no overall treatment effects ($P \geq 0.16$) for the CAR or COT SNP dose response curves (Figures 4.1B and 4.1D). In the CAR arteries, dietary treatment did not affect the pD_2 values ($P = 0.99$; pD_2: 8.14, 8.41, 8.26 ± 0.14 for MP60, MP80, and MP100, respectively). In the COT arteries, dietary treatment did not affect the pD_2 values ($P = 0.94$; pD_2: 8.07, 8.10, 8.13 ± 0.32 for MP60, MP80, and MP100, respectively).

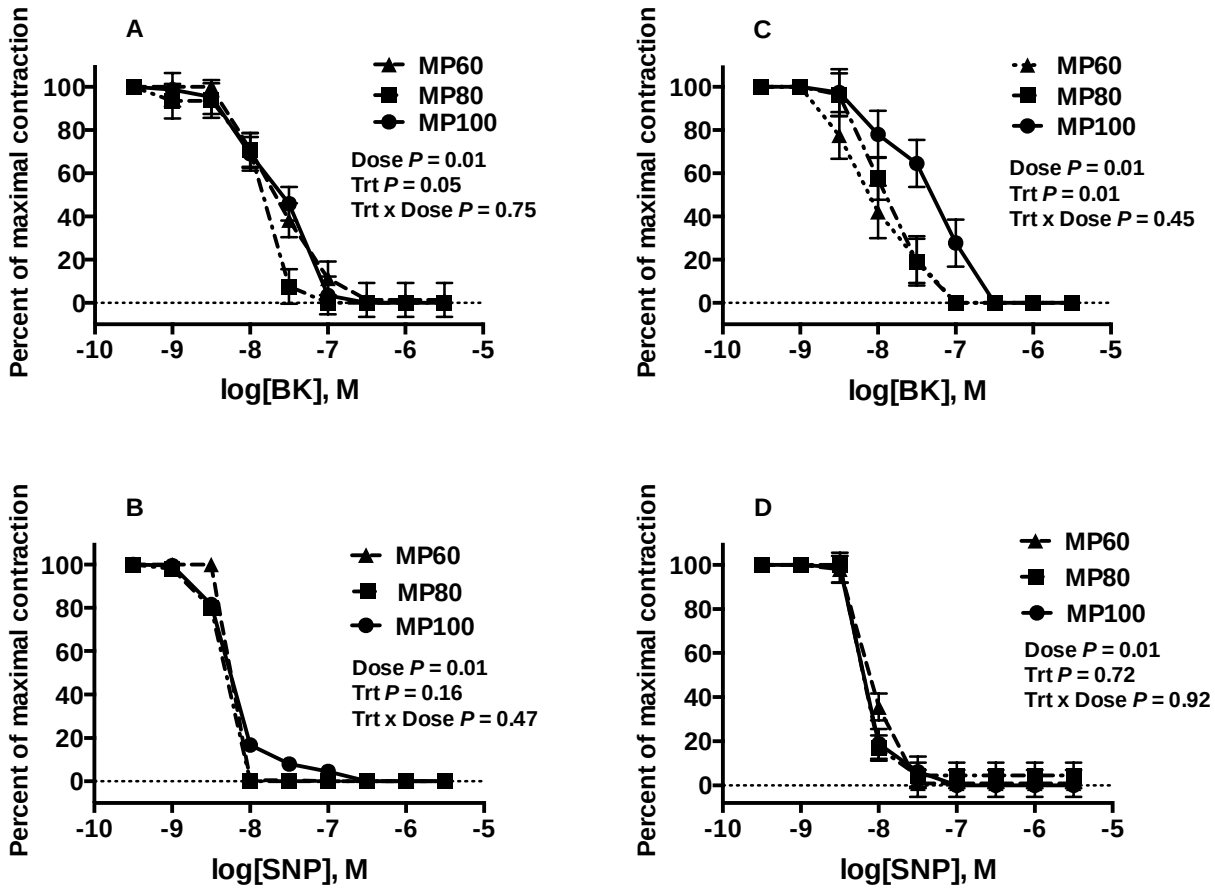

Figure 4.2. The effect of maternal metabolizable protein (**MP**) restriction on (**A**) the bradykinin (**BK**) dose response curve in caruncular (**CAR**) arteries, (**B**) the sodium nitroprusside (**SNP**) dose response curve in CAR arteries, (**C**) the BK dose response curve in cotyledonary (**COT**) arteries, and (**D**) the SNP dose response curve in COT arteries. Maternal diets were formulated to provide 60% of MP requirements (**MP60**), 80% of MP requirements (**MP80**), or 100% of MP requirements (**MP100**). Diets were fed from day 100 to 130 of gestation.

Potassium chloride (KCl) dose response curves. The results of the KCl dose response curves for CAR and COT arteries are visualized in Figures 4.3A and 4.3C, respectively). There was no treatment by endothelium by dose interactions ($P \geq 0.59$) on the KCl dose response curve for either the CAR or the COT arteries. In the CAR arteries, there was no treatment by dose interaction ($P = 0.98$) for the KCl dose response curve (Figure 4.3A). Further, there was no overall treatment effect ($P = 0.59$) on the KCl dose response curve in the CAR arteries (Figure 4.3A). Further, there were no treatment by endothelium, endothelium by dose (Figure 4.3A), or overall endothelium effects ($P \geq 0.58$) on the KCl dose response curve in the CAR arteries. In the COT arteries, there was a treatment by dose interaction ($P = 0.05$) on the KCl dose response curve. The MP60 ewes had a greater contraction ($P = 0.05$) to a lower dose of KCl compared with the MP80 and MP 100 ewes (Figure 4.3C), and the MP80 ewes had a greater contraction ($P = 0.05$) to a lower dose of KCl compared with the MP100 ewes (Figure 4.3C). However, after the 20mM dose of KCl, all COT arteries responded similarly to KCl regardless of maternal metabolizable protein level (Figure 4.3C). The endothelium by treatment interaction for the KCl dose response curve in the COT arteries was not statistically significant ($P = 0.75$; Figure 4.4A); however, there was an endothelium by dose interaction ($P = 0.01$) on the KCl dose response curve in the COT arteries (Figure 4.4B). The COT arterial rings that were denuded of the endothelium had a greater response ($P = 0.01$) to a low dose of KCl compared with the endothelium-intact arterial rings (Figure 4.4B). In the CAR arteries, dietary treatment did not affect the pD_2 values ($P = 0.99$; pD_2: 21.1, 21.5, 22.7 ± 1.41 for MP60, MP80, and MP100, respectively). Maternal diet also did not impact pD_2 values in the COT arteries ($P = 0.99$; pD_2: 19.9, 23.8, 29.9 ± 1.51 for MP60, MP80, and MP100, respectively).

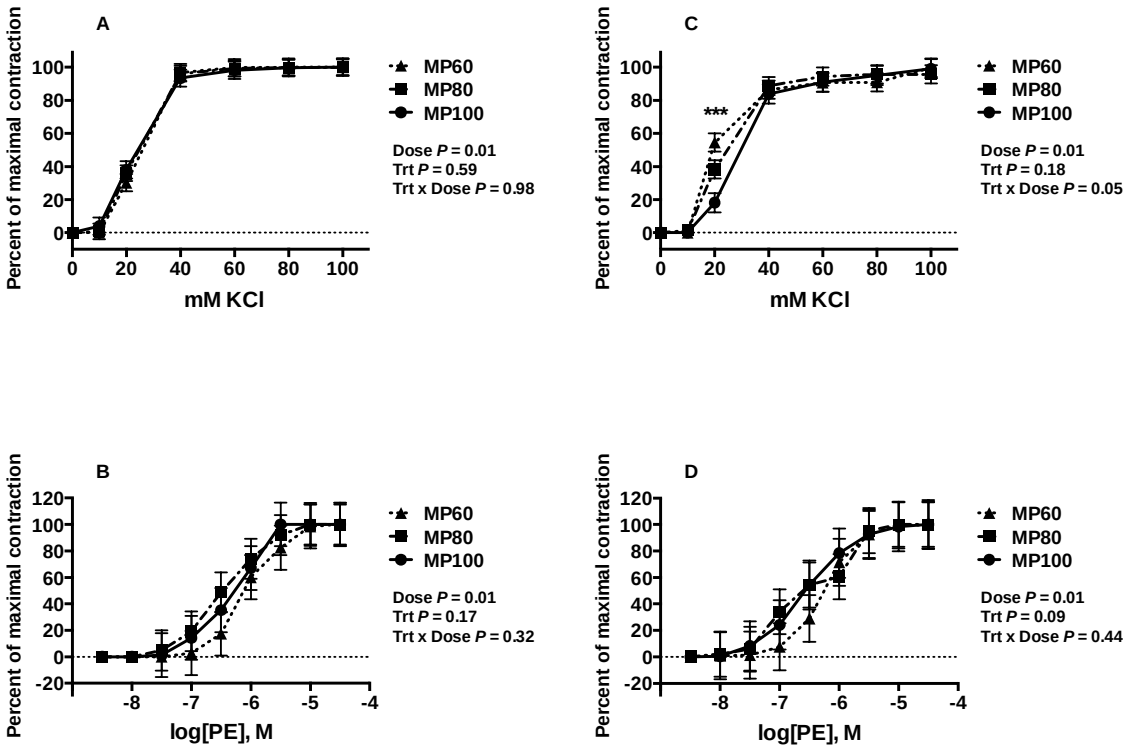

Figure 4.3. The effect of maternal metabolizable protein (**MP**) restriction on (**A**) the potassium chloride (**KCl**) dose response curve in caruncular (**CAR**) arteries, (**B**) the phenylephrine (**PE**) dose response curve in CAR arteries, (**C**) the KCl dose response curve in cotyledonary (**COT**) arteries, and (**D**) the PE dose response curve in COT arteries. Maternal diets were formulated to provide 60% of MP requirements (**MP60**), 80% of MP requirements (**MP80**), or 100% of MP requirements (**MP100**). Diets were fed from day 100 to 130 of gestation. *MP60 differs from MP80 and MP100 ($P \leq 0.05$) and MP80 differs from MP100 ($P \leq 0.05$).

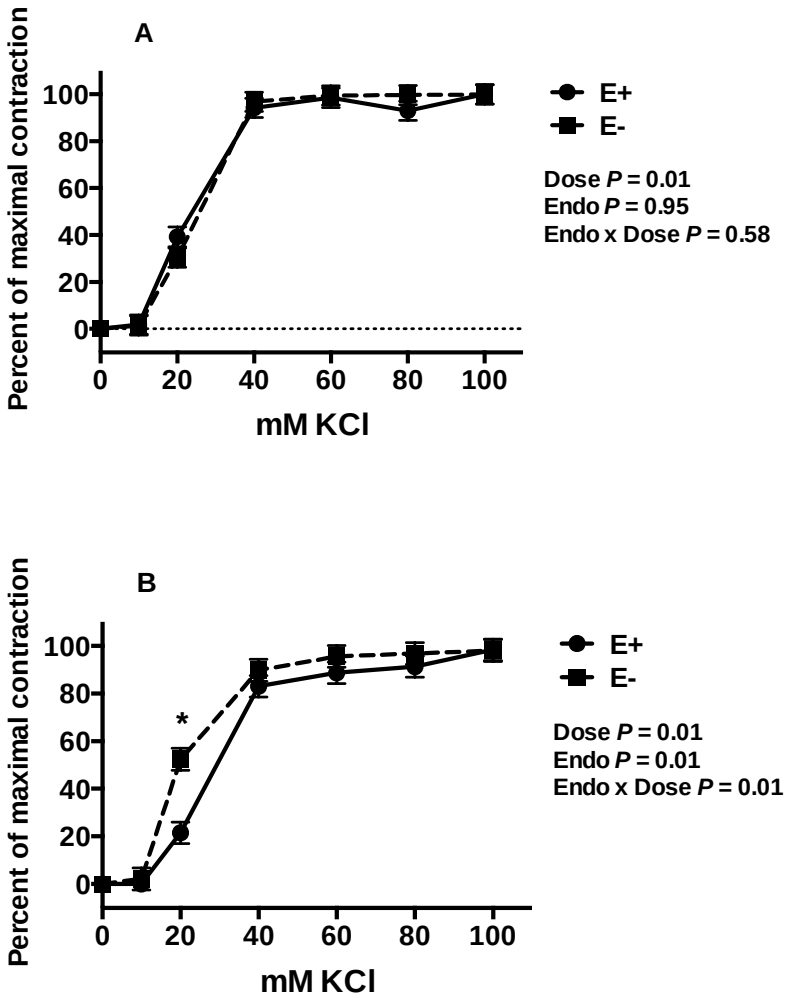

Figure 4.4. The effect of the presence (**E+**) or absence (**E-**) of endothelium on (**A**) the potassium chloride (KCl) dose response curve in caruncular arteries, (**B**) the KCl dose response curve in cotyledonary arteries. *E+ differs from E- ($P \leq 0.05$).

Phenylephrine (PE) dose response curves. The results of the PE dose response curves for CAR and COT arteries are visualized in Figures 4.3B and 4.3D, respectively. A treatment by endothelium by dose interaction on the PE dose response curve was not observed ($P \geq 0.30$) for either the CAR or the COT arteries. There were little evidence of treatment by dose interactions ($P \geq 0.32$) or overall treatment effects ($P \geq 0.09$) on the PE dose response curve for either the CAR or the COT arteries. Further, the treatment by endothelium and endothelium by dose interactions and the overall endothelium effect on the

PE dose response curve were not statistically significant ($P \geq 0.17$) for either the CAR or the COT arteries. In the CAR arteries, dietary treatment did not affect the pD_2 values ($P = 0.13$; pD_2: 6.08, 6.45, 6.30 ± 0.13 for MP60, MP80, and MP100, respectively). Maternal protein level also did not alter ($P = 0.35$) the pD_2 values in the COT (pD_2: 6.25, 6.51, 6.55 ± 0.18 for MP60, MP80, and MP100, respectively).

Endothelial nitric oxide synthase, GUCY1B3, BKR1, and BKR2 mRNA expression

Maternal metabolizable protein level did not significantly influence the mRNA expression of *eNOS* ($P = 0.79$; 1.60, 1.67, 1.48 ± 0.20 arbitrary units for MP60, MP80, and MP100, respectively), *GUCY1B3* ($P = 0.89$; 1.47, 1.50, 1.41 ± 0.14 arbitrary units for MP60, MP80, and MP100, respectively), *BKR1* ($P = 0.82$; 1.48, 1.37, 1.54 ± 0.21 arbitrary units for MP60, MP80, and MP100, respectively), or *BKR2* ($P = 0.74$; 1.65, 1.76, 1.98 ± 0.31 arbitrary units for MP60, MP80, and MP100, respectively) in the CAR arteries. Similarly, in the COT arteries, maternal metabolizable protein did not significantly influence the mRNA expression of *eNOS* ($P = 0.95$; 1.62, 1.57, 1.54 ± 0.18 arbitrary units for MP60, MP80, and MP100, respectively), *GUCY1B3* ($P = 0.89$; 1.61, 1.52, 1.62 ± 0.16 arbitrary units for MP60, MP80, and MP100, respectively), *BKR1* ($P = 0.91$; 1.37, 1.31, 1.45 ± 0.24 arbitrary units for MP60, MP80, and MP100, respectively), or *BKR2* ($P = 0.38$; 1.62, 1.33, 2.06 ± 0.44 arbitrary units for MP60, MP80, and MP100, respectively).

Discussion

This study aimed to investigate the effects of maternal metabolizable protein level on the vascular responses of the maternal and fetal placental arteries to two vasoconstrictors and two vasodilators. We hypothesized that a maternal diet low in protein would negatively impair the sensitivity of the placental arteries, particularly to BK, an endothelium-dependent vasodilator. We reject our hypothesis because placental arteries from ewes receiving the protein-restricted diet were more sensitive to BK compared to the placental arteries from control ewes. To our knowledge, there are limited data in the literature with regards to protein supplementation on ovine placental arteriole function, and this study provides insight into how the placental arteriole function may be altered by maternal protein levels.

While restricting MP during late gestation in sheep does not appear to negatively affect fetal weight or placental measurements at day 130 (current study), the present study suggests that decreasing

maternal MP may impact endothelium-dependent vascular function of the placenta. There was an overall treatment effect for the bradykinin dose response curves in both maternal and fetal placental arteries. The maternal placental arteries of the MP80 ewes were slightly more sensitive to BK compared with the MP60 and MP100 ewes. It is unclear why the placental arteries of MP80 ewes were more sensitive to BK-induced relaxation but the maternal placental arteries from the MP60 ewes did not show this same increased sensitivity. In the MP60 and MP80 fetal placental arteries were more sensitive to bradykinin-induced vasorelaxation compared with the MP100 ewes. Perhaps this increased sensitivity of the fetal placental arteries is a compensatory mechanism in the MP-restricted animals.

There is a limited information in the literature concerning how maternal nutrition of any kind, let alone protein amounts, impacts placental vascular function. We demonstrated that isocaloric diets with differing levels of MP during late gestation have effects on placental arterial responses to vasoconstrictors, an endothelium-dependent relaxant (BK) and an endothelium-independent relaxant (SNP). Relatively few studies exist that investigate the role of maternal protein deprivation and vascular responses to BK and SNP. One such study by Brawley et al. (2003) found that the mesenteric arteries from male offspring born to rats that were fed a 9% casein diet throughout gestation were less sensitive to BK-induced vasorelaxation at days 87 and 164 of life compared to males from rats fed an 18% casein diet. It is difficult to compare the current study to that of Brawley et al. (2003) for four reasons. First, different species were used in the two studies, and protein absorption in ruminants is different from that in rodents. Second, in the Brawley et al. (2003) study, the diets did consist of different levels of dietary protein, but no measures were taken to provide isocaloric diets as in the current study. Third, the current study investigated maternal and fetal placental arteries, whereas Brawley et al. (2003) studied the effects of protein restriction on the mesenteric artery. Finally, the current study investigated maternal and fetal placental arteries in near term sheep (day 130 of gestation), and Brawley et al. (2003) studied arteries from only male offspring that were in young adult and adult stages of life. Therefore, it is not surprising that Brawley et al. (2003) and the current study found different results when looking at BK-induced vasorelaxation. Ozaki et al. (2000) investigated the effects of global nutrient restriction (fed 50% of NRC requirements or 100% of NRC requirements) in pregnant ewes on the vascular response of small arteries from the fetal femoral bed to acetylcholine (an endothelium-dependent relaxant) on day 127 of gestation

and found that the restricted animals had a blunted response to ACh compared to the control animals (Ozaki et al., 2000). This data indicates that perhaps it is the net caloric intake that is resulting in the decreased response of arteries to endothelium-dependent relaxants (as observed in Ozaki et al., 2000 and Brawley et al., 2003) rather than protein restriction.

In the current study, maternal metabolizable protein level did not impact the maternal or fetal placental arterial response to endothelium-independent vasorelaxation (induced by SNP). Similar to their BK results, Brawley et al. (2003) found that the mesenteric arteries from male offspring born to rats that were fed a 9% casein diet throughout gestation were less sensitive to SNP-induced relaxation at days 87 and 164 of life compared with arteries from males from rats fed an 18% casein diet. Again, it is difficult to compare the current study with that of Brawley et al. (2003). Ozaki et al. (2000) investigated the effects of global nutrient restriction (fed 50% of NRC requirements or 100% of NRC requirements) in pregnant ewes on the vascular response of small arteries from the fetal femoral bed to SNP on day 127 of gestation and found that the restricted animals had a blunted response to SNP compared to the control animals (Ozaki et al., 2000). While Ozaki et al. (2000) utilized sheep, the design of the study was to investigate global nutrient restriction and not specifically protein deprivation. The differing results between Ozaki et al. (2000) and the current study are likely due to differences in the nutritional design of the two studies. Further, Ozaki et al. (2000) fed the restricted diets from day 0 to day 70 of gestation, whereas the current study fed MP-restricted diets from day 100 to 130 of gestation; therefore, comparisons between the two studies are difficult.

Brawley et al. (2003) also measured blood pressure of the male offspring at day 130 of life and found that males from dams that were protein restricted had an increase in blood pressure compared with males from dams that were not protein restricted. Based upon the blood pressure data and the BK and SNP results and, Brawley et al. (2003) concluded that maternal dietary protein restriction during gestation results in male offspring that were hypertensive due to vascular impairment of both endothelium-dependent and endothelium-independent vasorelaxation. The current study did not measure blood pressure, but the Brawley et al. (2003) results suggest that it is possible the maternal low protein diet could program the cardiovascular health of the offspring later in life, and if the current study were to have

collected day after day 130 of gestation, we may have observed hypertension in the offspring from protein-restricted dams.

In the current study, maternal placental arteries responded similarly to KCl, regardless of maternal MP level; however, in the fetal placental arteries, the initial contraction to KCl (20 mM) increased as maternal MP decreased, suggesting that maternal MP-restriction may result in increased hypertension in the fetal placental arteries. However, when taken together with the BK dose response curve data, it could be concluded that maternal MP-restriction may result in more sensitive fetal placental arteries, regardless if the stimulus is a relaxant or a constrictor. Maternal MP level did not influence the response to PE in either the maternal or the fetal placental arteries. Itoh et al. (2002) fed pregnant rats either a 9% casein diet or an 18% casein diet from day 0 to day 18 or 19 of gestation, at which time the vascular response of the uterine artery to KCl and PE was investigated. Itoh et al. (2002) did not find any differences between the two dietary groups for either vasoconstrictor. Again, it is difficult to directly compare the current study to that of Itoh et al. (2002) because of the difference in species, diet design, and arteries investigated, particularly because Itoh et al. (2002) examined the uterine artery whereas the current study examined the CAR and COT arteries.

In the present study, the fetal placental arteries were more sensitive to KCl when the endothelium was removed, but endothelium removal did not alter fetal placental responses to PE. This is similar to Tsuji et al. (1994) who found that removing the endothelium potentiated the contractile response to KCl but had no effect on the contractile response to PE. This indicates that while KCl acts upon vascular smooth muscle cells, the endothelium plays an important role in modulating the calcium influx into the smooth muscle cells.

The mRNA expression of the BK receptors 1 and 2 (BKR1 and BKR2) was analyzed in the present study. Because one of the mechanism of BK-induced relaxation involves nitric oxide, the mRNA of NO and it's receptor soluble guanylate cyclase was also analyzed. Since the current study observed an overall treatment effect on the maternal and fetal placental arterial response to BK, one might expect to see differences among the three MP diets in mRNA expression of BK receptors 1 and 2 in these arteries; however, this was not the case. The mRNA expression of all genes investigated was similar among all treatments in both maternal and fetal placental arteries. This is an important finding, as it

indicates that even though gene expression was not altered, vascular function was, and it supports the need for more vascular function studies.

In the present study, feeding diets formulated to provide 60% of MP resulted in decreased ewe body weight throughout gestation compared with ewes fed 80% of MP as well as a lower percentage change in BW compared to ewes receiving either 80% or 100% of MP. Van Emon et al. (2013) fed similar diets to a larger group of ewes (n = 295) and found that by lambing, ewe BW and percentage change in BW increased linearly as MP level increased from 60% to 100%. It is possible that a linear effect on ewe BW and the percentage change in ewe BW would have been observed in the current study if a larger sample size was used or if the ewes were fed the diets through gestation.

Maternal undernutrition has been shown to cause fetal growth restriction in sheep (Charlton and Johengen, 1985; Holst et al., 1986; Faichney and White, 1987; Holst et al., 1992; Bloomfield et al., 2000; Vonnahme et al., 2003; Scheaffer et al., 2004; Lekatz et al., 2010a, 2010b; Lemley et al., 2012). In the current study, maternal MP level did not alter fetal weight, length or girth, which indicates that fetal mass and size is more dependent on the overall amount of calories received rather than the level of MP in the maternal diet. When isocaloric diets that differed in the level of MP were fed to ewes from day 100 to lambing, there was no difference in lamb birth weight (Van Emon et al., 2013), which indicates that even if the MP treatments are fed through lambing, there are no negative effects on fetal weight. Also of importance, decreasing the MP level to 60% or 80% of NRC requirements did not alter the percentage of assisted births or the percentage of lambs requiring treatment for illness at birth, which also indicates that decreasing MP level during gestation does not have negative effects on birthing or neonatal health (Van Emon et al., 2013). Therefore, neonatal health and performance does not appear to be influenced by maternal protein levels in the diet when fed at or above 60%. However, others have shown enhanced phenotypes in offspring born from dams that were supplemented with protein during the last third of gestation. For example, Stalker et al. (2006) found that calves born to cows that were supplemented with crude protein during late gestation were heavier at weaning than calves born to cows that were not supplemented. So while, we did not observe any differences due to diet on fetal weight and Van Emon et al. (2013) did not observe differences due to diet on lamb weight or health, the Stalker et al. (2006) data indicates that differences to the offspring may not be observed until later in life.

Maternal nutrient restriction in sheep has also been linked to altered placental weight in some studies (Charlton and Johengen, 1985; Faichney and White, 1987; McCrabb et al., 1991, 1992a, 1992b; Heasman et al, 1998; McMullen et al., 2005) but not in others (Vonnahme et al., 2003; Scheaffer et al., 2004; Holst et al., 1992; Bloomfield et al., 2000; Lemley et al., 2012; Lekatz et al., 2010a, 2010b). It is likely that the effects of maternal nutrition on placental characteristics vary among studies due to the timing and severity of nutrient restriction. In the current study, diets were "adequate in calories", however, maternal MP level from day 100 to 130 of gestation did not alter placental measurements. However, weight is not always a good indicator of function.

In conclusion, this study investigates the role of maternal MP restriction from day 100 to day 130 of gestation on the vascular function of maternal and fetal placental arteries in near term sheep. The lack of differences in fetal and placental measurements indicates that not meeting MP requirements does not negatively impact fetal or placental growth in fetal sheep. However, MP level did influence vascular function of placental arteries. Metabolizable protein restriction resulted in increased sensitivity to an endothelium-dependent relaxant in fetal placental arteries. This may be a compensatory mechanism of the protein restricted animals, and this could be a beneficial result of low MP. Further studies are needed to better understand how maternal MP alters placental vascular function, what mechanisms are responsible for the endothelium-dependent vasorelaxation of the placental arteries, and how maternal MP might influence the mechanisms of vasorelaxation.

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CHAPTER 5. BRADYKININ-INDUCED VASORELAXATION IS ACHIEVED THROUGH DIFFERENT MECHANISMS IN MATERNAL AND FETAL PLACENTAL ARTERIES IN SHEEP

Abstract

We hypothesize that restricting or overfeeding maternal metabolizable protein (**MP**) during late gestation will increase the sensitivity to bradykinin (**BK**)-induced vasorelaxation in the fetal placental arteries. Further, we hypothesize maternal MP level will influence the mechanism of BK-induced vasodilation in placental arteries. Pregnant ewes (n = 18) were assigned to one of three isocaloric diets formulated to provide 60%, 100%, or 140% of the MP requirements (**MP60**, **MP100**, and **MP140**, respectively) from day 100 to 130 of gestation. Pharmacological studies investigated the dose response curves (**DRCs**) to BK in caruncular (**CAR**) or cotyledonary (**COT**) arteries in the absence or presence of one of three inhibitors: iberiotoxin (inhibits K+ channels), indomethacin (inhibits cyclooxygenase 1 and 2, **COX-1** and **-2**) or *N*(omega)-L-arginine methyl ester (inhibits nitric oxide synthase, **NOS**). There were no treatment by dose interactions for any of the DRCs ($P \geq 0.79$) in CAR or COT arteries. In CAR arteries, inhibiting K+ channels and NOS resulted in a rightward shift ($P = 0.01$) of the BK DRC, indicating that both of these mechanisms are important for BK-induced vasodilation in CAR arteries. However, inhibition of COX-1 and -2 failed to attenuate ($P = 0.89$) the BK response, indicating that prostacyclin may not be involved in BK-responses in CAR arteries. In the COT arteries, there was a leftward shift ($P = 0.01$) in the BK DRC for MP60 and MP140 ewes compared with MP100 ewes, indicating that both maternal MP restriction and excess results in increased sensitivity to BK in COT arteries. Inhibition of K+ channels, COX-1 and -2, and NOS failed to prevent vasorelaxation in COT arteries as all E_{max} values were similar ($P = 0.11$) to the BK DRC with no inhibitor. Because COT arteries relaxed in the presence of these inhibitors, BK-induced vasodilation in COT arteries in the current study must be achieved through an unclassified endothelium-dependent relaxing factor.

Key words: bradykinin, ewes, metabolizable protein, pregnancy, vascular function

Introduction

Increasing uterine blood flow during the last half of gestation is critical for oxygen and nutrient delivery to the exponentially growing fetus, and alterations to placental blood flow vasculature may compromise the growth of the developing fetus (Ford, 1995; Meschia, 1983; Redmer et al., 2004; Reynolds et al., 2005; Reynolds et al., 2006). In sheep, maternal nutrient restriction reduces uterine (Lemley et al., 2012) and umbilical (Lemley et al., 2012; Lekatz, Chapter 2) blood flow.

An important question to consider is if these alterations to the placenta and blood flow are due to an overall caloric restriction or to restriction of a specific component in the diet. Evidence in rodent models suggests that maternal dietary protein level plays an important role in vascular tone of pregnant animals. For example, protein restriction during pregnancy impairs vascular relaxation of peripheral arteries (Koumentaki et al., 2002) and the uterine artery (Itoh et al., 2002) in rats, which could negatively affect blood flow to the developing fetus. Many of the rodent models investigating maternal protein restriction investigate vascular responses to acetycholine (Itoh et al., 2002; Koumentaki et al., 2002; Torrens et al., 2002; Musha et al., 2006), but bradykinin-induced vasoresponses may be of more importance, especially when considering hypertension in the offspring (Wirth et al., 1996) and bradykinin's close association with the renin angiotensin system.

Our laboratory found that restricting maternal metabolizable protein (**MP**) during late gestation resulted in increased sensitivity to bradykinin in the fetal placental cotyledonary arteries near term in sheep (Lekatz, Chapter 3). This increased sensitivity of placental arteries to bradykinin may be a compensatory mechanism in sheep not meeting MP requirements. The exact mechanisms of bradykinin-mediated vasodilation in placental arteries of sheep are not known. Further how maternal metabolizable protein level might impact these mechanisms has not been investigated. Therefore, the objective of this study was to examine the effect of three levels of maternal MP during late gestation on the mechanisms of bradykinin-induced vasorelaxation in the placental arteries of sheep.

Materials and methods

Animal studies were approved the by North Dakota State University Animal Care and Use Committee. On day 90 of gestation, 18 pregnant multiparous ewes carrying a single fetus, were housed in

individual pens (0.91 × 1.2 m) in an indoor facility until necropsy (130 ± 2 days of gestation). Temperature was held constant at 12ºC, and lighting was controlled automatically (12:12-h light-dark cycle with lights on at 07:00 and of at 19:00).

Ewes were acclimated to low-quality hay (Table 5.1) and the MP100 supplement (100% of the MP requirements, as determined by NRC, 2007; Table 2) for 10 days prior to starting dietary treatments. Ewes were weighed on two consecutive days (days 99 and 100 of gestation) prior to the initiation of treatments. On day 100 of gestation ewes were randomly assigned to one of three isocaloric dietary treatments (Table 5.2): **MP60**: 60% of MP requirements, **MP100**: 100% of MP requirements, and **MP140**: 140% of the MP requirements on a DM basis during the last 4 weeks of gestation (NRC, 2007). Dietary treatments were fed from 100 to 130 days of gestation. Metabolizable protein intake was determined by the average of the days 99 and 100 body weight and offered once daily at 0700 h. Ewes were given one hour to consume the supplement then low-quality forage (Table 5.1) was offered. Body weights were collected every 10 days throughout the dietary treatment period, and the amount of supplement and low-quality forage offered was adjusted for changes in body weight. All ewes had access to fresh water and trace mineralized salt (4,000 ppm Zn, 1,600 ppm Fe, 1.200 ppm Mn, 325 ppm Cu, 100 ppm I, 40 ppm Co; American Stockman, Overland Park, KS).

Table 5.1. Nutrient composition of fescue straw[1].

Item	
Diet, % DM	56.51
DM, %	77.61
NEm, Mcal/kg	2.12
CP, % of DM	3.07
MP, % of DM	1.97
NDF, % of DM	81.13
ADF, % of DM	51.10
Ash, % of DM	7.78

[1]Ewes were fed fescue straw to limit MP intake

Table 5.2. Ingredient and nutrient composition of dietary supplements fed to ewes.

Item	Treatment[1]		
	MP60	MP100	MP140
Ingredient, % DM			
Corn	30.00	19.00	5.00
DDGS[2]	4.00	24.00	30.00
Soyhulls	9.00	—	—
Nutrient composition			
DM, %	88.64	90.19	92.16
NEm, Mcal/kg	2.05	2.19	2.06
CP, % of DM	10.21	18.67	28.68
MP, % of DM	6.54	11.96	18.37
NDF, % of DM	29.64	31.40	45.34
ADF, % of DM	13.87	8.68	13.34
Ash, % of DM	3.53	3.50	5.13

[1]Maternal diets (DM basis) were balanced for mature ewes baring a singleton during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60: 60% of MP requirements; MP100: 100% of MP requirements; and MP140: 140% of MP requirements.

[2]Dried distillers grains with solubles

Tissue collection and fetal and placental measurements

On day 130 of gestation, ewes were weighed and euthanized by captive bolt (Supercash Mark 2, Accles and Shelvoke Ltd., Sutton Coldfield, West Midlands, UK) followed by exsanguination. The gravid uterus was immediately dissected cranial to the cervix and weighed. The uterus was opened along the antimesometrial side, the umbilical cord was ligated, and the fetus was removed, weighed, and fetal curved crown rump and abdominal girth were recorded. Immediately after the fetus was removed, the terminal branches of fetal placental arteries (cotyledonary arteries; **COT** arteries) were dissected and either immersed in cold physiological salt solution for the organ bath chamber study (described below) or snap frozen in liquid nitrogen and stored at -80ºC for qPCR (described below). These COT arteries, terminating directly into the cotyledon, were typically secondary branches of the umbilical artery. Following removal of the fetal placental arteries, the gravid uterine artery was located and maternal placental arteries (caruncular arteries; **CAR** arteries) terminating at the caruncle were dissected and dissected and either immersed in cold physiological salt solution for the organ bath chamber study (described below) or snap frozen in liquid nitrogen and stored at -80ºC for qPCR (described below).

These CAR arteries, terminating directly into the caruncle, were typically 4th or 5th branches of the gravid uterine artery. Because placentome size has been shown to influence vascular reactivity (Vonnahme et al., 2008) similarly sized placentomes were selected for each animal.

Next, the fetal fluid was collected and the volume recorded. Placentomes were counted, dissected, weighed, and the CAR and COT tissues were separated and weighed. The fetal membranes and empty uterine weight were also weighed.

Quantification of mRNA

The relative mRNA expression of endothelial nitric oxide synthase (**eNOS**), soluble guanylate cyclase (**GUCY1B3**), bradykinin receptor 1 (**BKR1**), and bradykinin receptor 2 (**BKR2**) was determined in the CAR and COT arteries. Caruncular and COT arteries (30mg) were homogenized using a Polytron homogenizer fitted with a 7mm generator. Messenger RNA was isolated using a QIAshredder spin column and the RNeasy Mini Kit as described by the manufacturer (Qiagen, Valencia, CA). RNA was quantified by measuring the absorbance on a Nanodrop 2000c spectrophotometer. Synthesis of cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit as described by the manufacturer (Qiagen). This protocol includes a genomic DNA removal step, which was performed for all samples. Quantitative PCR (qPCR) reactions for eNOS and sGC contained the following: 1x TaqMan Universal PCR Master Mix (Life Technologies), forward and reverse primers (each 1μM), water and cDNA for a final volume of 12.5 μl. Reactions to detect BKR1 and BKR2 contained the following: 1X SYBR Green PCR Master Mix (Life Technologies), forward and reverse primers (each 500 nM), water and cDNA for a final volume of 12.5 μl. Dissociation curves confirmed a single amplification species for both BKR1 and BKR2. Amplification of 18s rRNA using a 20X predesigned assay reagent, which contained both forward and reverse primers and a probe (Life Technologies), was performed for each sample and was used to normalize expression of each gene. The ratio of the gene of interest to 18S rRNA was used for quantifying the gene expression and is the data presented.

Organ chamber studies

After transfer to the laboratory, the CAR and COT arteries were dissected free from surrounding placental tissue, cleaned of adherent fat and connective tissue, and cut into rings either 2-3 mm in length (CAR arteries) or 4-5 mm in length (COT arteries). Four rings were prepared from both CAR and COT arteries for each ewe. The endothelium remained intact in all of the arterial rings.

The following drugs were used: KCl, norepinephrine, bradykinin, indomethacin, *N*(omega)-L-arginine methyl ester, iberiotoxin (Sigma Chemical, St. Louis, MO), and 9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (Cayman Chemical, Ann Arbor, MI). Drug solutions were prepared daily, kept on ice, and protected from light until used. All drugs were dissolved initially in double-distilled water. Drug concentrations are reported as final molar concentrations in the organ chamber. The composition of the physiological salt solution was (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 11.1 glucose.

Caruncular (n = 4 for each ewe) rings were mounted onto a wire myograph chamber (DMT-USA, Inc., Ann Arbor, MI) by passing two 20 µm steel wires through the lumen and secured to either side via supporting jaws. The chambers were aerated with 95% O_2/5% CO_2 and the temperature was maintained at 37˚C throughout the experiment. Isometric tension was measured and recorded using a PowerLab data acquisition system. The tissues were stretched progressively to the optimal point of their length-tension relationship, using KCl (100mM) to generate a standard contractile response. After this procedure, the rings were allowed to equilibrate at their optimal length-tension for one hour prior to further exposure to any vasoactive substances. The presence of intact endothelium was confirmed in each preparation by contracting the rings with norepinephrine (**NE**; 10^{-6} M) and observing the presence or absence of relaxation to the endothelium-dependent vasodilator, bradykinin (**BK**; 10^{-7} M).

Cotyledonary arterial rings (n = 4 for each ewe) were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution, as previously described (Tunstall et al., 2011; Shukla et al., 2012). The chambers were aerated with 95% O_2/5% CO_2 and the temperature was maintained at 37˚C throughout the experiment. Each ring was suspended by means of two fine stainless-steel wire clips passed through the lumen; one clip was anchored inside the organ chamber, the other connected to a force transducer (Model FT03, Grass Instrument Company, Quincy, MA, USA). Isometric

tension was measured and recorded on a Grass polygraph. The tissues were stretched progressively to the optimal point of their length-tension relationship, using KCl (100mM) to generate a standard contractile response. After this procedure, the rings were allowed to equilibrate at their optimal length-tension for one hour prior to further exposure to any vasoactive substances. The presence of intact endothelium was confirmed in each preparation by contracting the rings with 9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (**U46619**; 10^{-6} M) and observing the presence or absence of relaxation to the endothelium-dependent vasodilator, bradykinin (**BK**; 10^{-7} M).

The same dose response curves were obtained in CAR and COT arteries. Briefly, one ring underwent a BK dose response curve in the absence of any inhibitor (no inhibitor, **NI**) and the remaining rings underwent a BK dose response curve in the presence of one of three inhibitors, iberiotoxin (**IBTX**), indomethacin (**INDO**), or *N*(omega)-L-arginine methyl ester (**NLA**).

A BK dose response curve in the absence of an inhibitor was obtained in one CAR and one COT arterial ring. The rings were contracted with U46619 (10^{-8} M); after the U46619-induced contraction had reached a stable plateau, relaxation responses to increasing concentrations of the endothelium-dependent vasodilator BK (10^{-10} to 10^{-6} M) in the absence of an inhibitor (no inhibitor, NI) were obtained. The remaining rings were incubated with one of three inhibitors: IBTX (10^{-7} M), INDO (10^{-5} M), or NLA (10^{-5} M) for 30 minutes before being contracted with U46619 (10^{-8} M). After the U46619-induced contraction had reached a stable plateau, relaxation responses to increasing concentrations of the endothelium-dependent vasodilator BK (10^{-10} to 10^{-6} M) were obtained.

Data analysis

Relaxation responses are expressed as a percentage of the initial tension induced by U46619 or NE. For each vasoactive agent, both the maximal percent response (E_{max}) and the concentration necessary to produce 50% of its own maximal response (EC_{50}) were determined using Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Briefly, the log[M concentration] was plotted along the x-axis and percent of maximal contraction plotted along the y-axis. Next, Prism 6 software was used to find the best fit curve using nonlinear regression with the dose response equation for log(dose) vs. response. The EC_{50} values were converted to the negative logarithms and expressed as -log molar EC_{50} (pD_2). Initial

and final ewe body weight, fetal measurements, placental measurements [including the average placentome weight, calculated as: (total placentome weight / placentome number)], the fetal weight to placental weight [calculated as: (fetal weight / total placentome weight)], qPCR data, and E_{max} were analyzed by ordinary least squares [GLM procedure of SAS (SAS software version 9.2; SAS Institute, Cary, NC)], with treatment in the model statement. The pD_2 values were analyzed using Prism 6 software (GraphPad Software, Inc.). The dose response curves were also analyzed using ordinary least squares [GLM procedure of SAS (SAS Institute)], with treatment, dose, and inhibitor main effects, treatment by dose, dose by inhibitor, and treatment by inhibitor by dose interactions in the model statement. Within each treatment, the dose response curves were analyzed using ordinary least squares [GLM procedure of SAS (SAS Institute)] with dose and inhibitor main effects and the dose by inhibitor interaction in the model statement. For both maternal and fetal placental arteries, the overall inhibitor dose response curves were compared to the dose response curve with no inhibitor using ordinary least squares [GLM procedure of SAS (SAS Institute)] with dose and inhibitor main effects and the dose by inhibitor interaction in the model statement. For all data, if the F-test was significant, means were separated using least significant difference. Least squares means and SE are reported.

Results

Ewe body weight

Initial ewe body weight did not differ ($P = 0.98$) among treatments (61.9, 63.1, and 62.6 ± 4.23 kg for MP60, MP100, and MP140, respectively). Maternal MP level also did not affect ($P = 0.80$) ewe weight at slaughter (63.4, 67.5, and 66.0 ± 4.33 kg for MP60, MP100, and MP140, respectively) or the percentage change in ewe BW at slaughter ($P = 0.06$; 0.01, 0.03, and 0.01 ± 0.001 % for MP10, MP100, and MP140, respectively). There was a treatment by day interaction ($P = 0.02$) on ewe BW throughout gestation (Figure 5.1); however, when the means were separated (PDIFF option of LSMEANS statement; SAS, Inc.), no *P*-values were less than 0.05.

Fetal and placental measurements

Fetal weight, fetal curved crown rump, and fetal girth were not influenced ($P \geq 0.50$) by maternal metabolizable protein level (Table 5.3). Gravid uterine weight, empty uterine weight, total placentome weight, total placentome number, average placentome weight, total CAR weight, total COT weight, fetal membrane weight, fetal fluid volume, and fetal weight / placentome weight were also not influenced ($P \geq 0.09$) by maternal metabolizable protein level (Table 5.3).

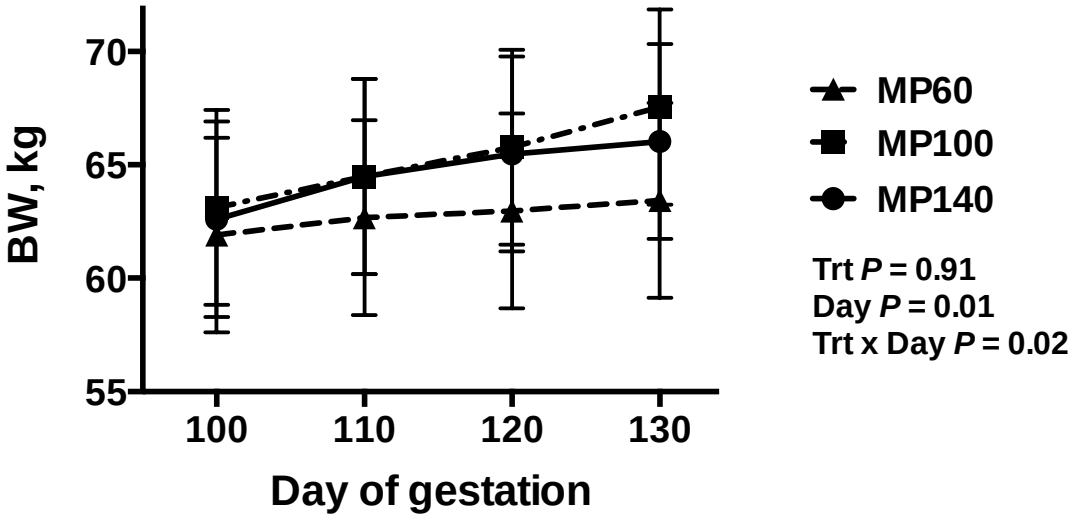

Figure 5.1. The effect of maternal metabolizable protein (**MP**) level on ewe body weight throughout gestation. Maternal diets were formulated to be isocaloric and provide 60% of MP requirements (**MP60**), 100% of MP requirements (**MP100**), or 140% of MP requirements (**MP140**). Diets were fed from day 100 to 130 of gestation.

Organ bath studies

Caruncular artery bradykinin dose response curves in the absence or presence of an inhibitor. There were no interactions ($P \geq 0.79$) on the BK dose response curves in COT arteries with no inhibitor (Figure 5.2A) or for COT arteries incubated with IBTX (Figure 5.2B), INDO (Figure 5.2C), or NLA (Figure 5.2D). There was no treatment by dose by dose response curve interaction ($P = 0.99$), so the dose by dose response curve interaction, dose main effect, and dose response curve main effect were analyzed for each treatment (MP60, MP100, and MP140) individually (Figure 5.3). In the MP60 ewes, there was a

dose by dose response curve interaction (P = 0.02), where the CAR placental arteries responded similarly to BK in the presence or absence of any inhibitor used (IBTX, INDO, or NLA) until the 3×10^{-8} M dose. After this dose, the MP60 CAR arteries without an inhibitor were more sensitive (P = 0.02) to BK compared with the CAR arteries incubated with IBTX or NLA for the remaining doses of BK (Figure 5.3A). The CAR arteries incubated with INDO responded similarly to CAR arteries with no inhibitor at the 3×10^{-8} M, 10^{-7} M, and 3×10^{-6} M doses (Figure 5.3A). In the MP100 ewes, the dose by treatment interaction was not significant (P = 0.13), but it appears CAR arteries incubated with INDO responded similarly to CAR arteries without an inhibitor (Figure 5.3B). Further, it appears that incubation with either IBTX or NLA delayed the BK-induced relaxation compared with the CAR arteries with no inhibitor (Figure 5.3B).

Table 5.3. The effect of maternal metabolizable protein level on gravid uterine weight, total fetal weight, average fetal weight, fetal curved crown length, fetal girth, total placentome weight, total placentome number, average placentome weight, total caruncular weight, total cotyledonary weight, fetal membrane weight, fetal fluid volume, empty uterine weight, and fetal weight to placental weight ratio.

	Treatments[1]				
Measurement	MP60	MP80	MP100	SE	*P*-value
Gravid uterine weight (g)	9277	9756	9348	1097	0.95
Total fetal weight[2] (g)	2450	2908	2602	445	0.80
Average fetal weight (g)	2110	2485	2187	443	0.79
Fetal CCL[3] (cm)	54.6	58.7	53.1	2.42	0.24
Fetal girth (g)	32.6	33.8	31.3	1.60	0.51
Total placentome weight[4] (g)	754.1	780.8	774.3	60.9	0.93
Total placentome number	80.0	89.5	79.7	7.10	0.50
Average placentome weight (g)	9.24	9.16	9.76	1.19	0.91
Total CAR[5] weight (g)	123.3	126.5	122.5	12.7	0.97
Total COT[6] weight (g)	573.7	595.9	592.4	47.7	0.92
Fetal membrane weight (g)	336.9	347.5	333.1	42.4	0.97
Fetal fluid volume (mL)	2212	2304	2299	298	0.97
Empty uterine weight (g)	66937	782.1	773.3	70.8	0.38
Fetal weight/total placentome weight (g/g)	4.10	4.04	3.78	0.83	0.94

[1]Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments: **MP60**: 60% of MP requirements; **MP80**: 80% of MP requirements; and **MP100**: 100% of MP requirements.
[2]Total fetal weight = total fetal weight per ewe
[3]Fetal CCL = fetal curved crown length
[4]Total placentome weight = total placentome weight per ewe
[5]CAR = caruncular
[6]COT = cotyledonary

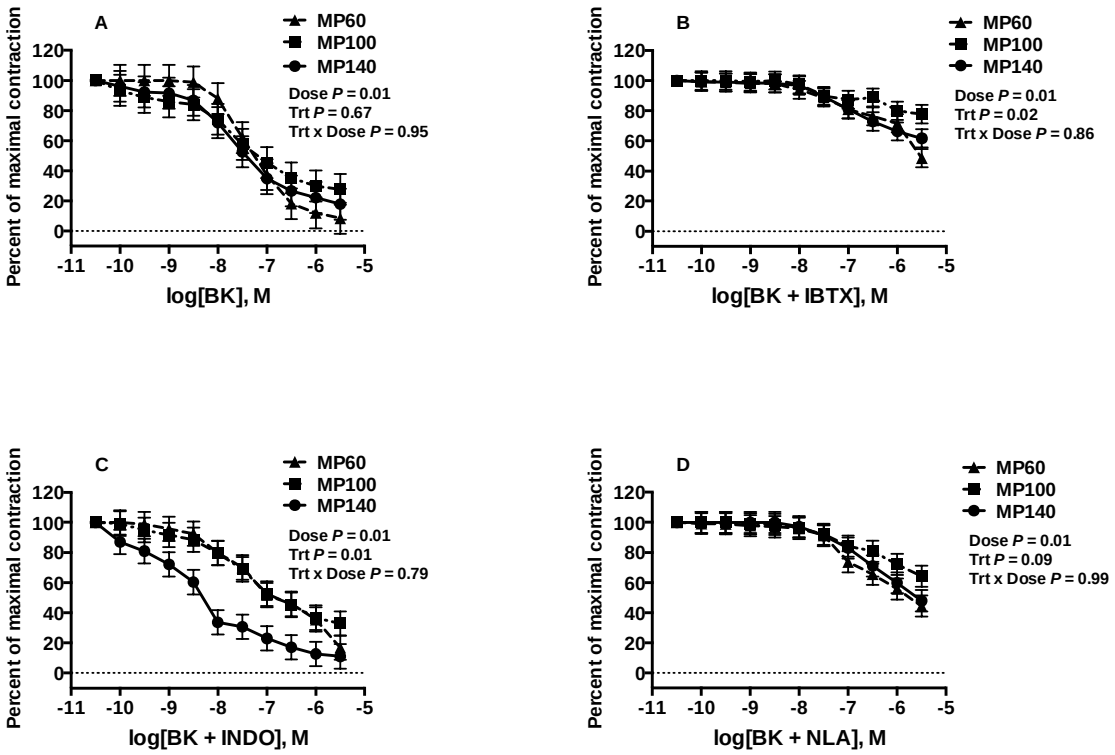

Figure 5.2. The interaction of maternal metabolizable protein (**MP**) level and bradykinin (**BK**) dose on the BK dose response curve in caruncular arteries (**A**) in the absence of an inhibitor (no inhibitor, **NI**), (**B**) after incubation with iberiotoxin (**IBTX**), (**C**) after incubation with indomethacin (**INDO**), and (**D**) after incubation with *N*(omega)-L-arginine methyl ester (**NLA**). Maternal diets were formulated to be isocaloric and provide 60% of MP requirements (**MP60**), 100% of MP requirements (**MP100**), or 140% of MP requirements (**MP140**). Diets were fed from day 100 to 130 of gestation.

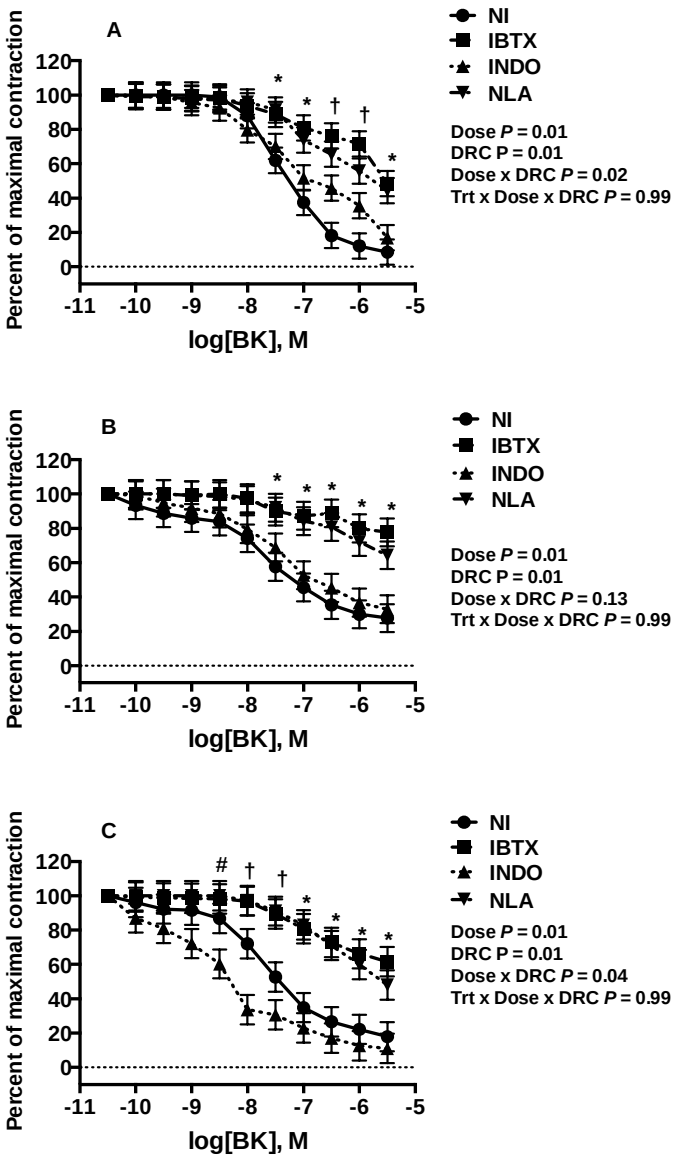

Figure 5.3. The effect of dose response curve (**DRC**) in caruncular (**CAR**) arteries on the bradykinin (**BK**) DRC within each dietary treatment. Ewes were fed diets formulated to provide (**A**) 60% of metabolizable protein (**MP**) requirements (**MP60**), (**B**) 100% of MP requirements (**MP100**), and (**C**) 140% of MP requirements (**MP140**). The CAR arteries were incubated with no inhibitor (**NI**) or incubated with iberiotoxin (**IBTX**), indomethacin (**INDO**), or *N*(omega)-L-arginine methyl ester (**NLA**). Diets were fed from day 100 to 130 of gestation. †NI is statistically different from IBTX, NLA, and INDO ($P \leq 0.05$). *****NI is statistically different from IBTX and NLA ($P \leq 0.05$). #NI is statistically different from INDO ($P \leq 0.05$).

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In the MP140 ewes, there was a dose by dose response curve interaction (Figure 5.3C). The CAR arteries incubated with INDO were more sensitive ($P = 0.04$) to BK at the 3×10^{-9} M, 10^{-8} M, and 3×10^{-8} M doses compared with arteries with no inhibitor (Figure 5.3A). Beginning at the 10^{-8} M dose of BK, CAR arteries that were not incubated with an inhibitor were more sensitive ($P = 0.04$) to the remaining doses of BK compared with the CAR arteries incubated with either IBTX or NLA (Figure 5.3C).

When all treatments were combined, there was also a dose by dose response curve interaction on the BK dose response curves (Figure 5.4A). Beginning at the 3×10^{-8} M dose, CAR arteries with no inhibitor were more sensitive ($P = 0.01$) to BK compared with CAR arteries in the presence of IBTX or NLA (Figure 5.4A). The CAR arteries incubated with INDO were more sensitive ($P = 0.01$) to the 3×10^{-8} M dose of BK compared with the arteries in the absence of an inhibitor, but after this dose, the CAR arteries incubated with INDO responded to BK similarly ($P = 0.01$) to CAR arteries with no inhibitor (Figure 5.4A).

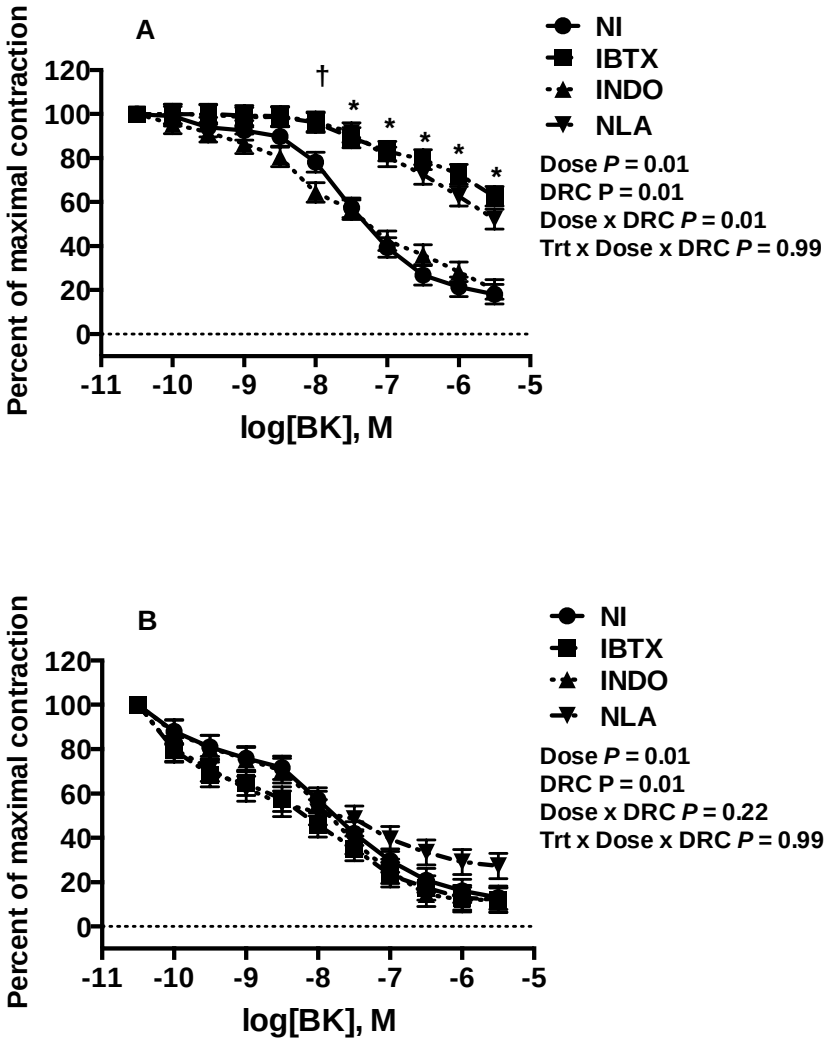

Figure 5.4. The effect of dose response curve (**DRC**) on the bradykinin (**BK**) DRC in (**A**) in caruncular and (**B**) cotyledonary arteries. The CAR arteries were incubated with no inhibitor (**NI**) or incubated with iberiotoxin (**IBTX**), indomethacin (**INDO**), or *N*(omega)-L-arginine methyl ester (**NLA**). †NI is statistically different from IBTX, NLA, and INDO ($P \leq 0.05$). *****NI is statistically different from IBTX and NLA ($P \leq 0.05$).

Caruncular artery EC_{50}, pD_2, and E_{max} values. Maternal MP level did not impact ($P = 0.46$) the pD_2 value for the BK dose response curve in CAR arteries with no inhibitor (7.20, 7.00, 7.28 ± 1.78 for MP60, MP100, and MP140, respectively). In CAR arteries incubated with IBTX, the pD_2 value was lower ($P \leq 0.04$) in the MP100 ewes compared with the MP60 and MP140 ewes, which were similar ($P = 0.38$;

3.98 vs. 5.41 and 5.15 ± 0.74 for MP100, MP60, and MP140, respectively). In CAR arteries incubated with INDO, the MP140 ewes had a greater (P = 0.01) pD_2 value compared to both MP60 and MP100 ewes, which were similar (P = 0.53; 8.21 vs. 6.72 and 6.60 ± 0.15 for MP140, MP60, and MP100, respectively). In CAR arteries incubated with NLA, the MP100 ewes had a lower ($P \leq 0.03$) pD_2 value compared with MP60 and MP140 ewes, which were similar (P = 0.45; 4.95 vs. 5.80 and 5.64 ± 0.34 for MP100, MP60, and MP140, respectively). The BK dose response pD_2 values for CAR arteries with no inhibitor and CAR arteries incubated with INDO were similar (P = 0.89; 7.17 and 7.16 ± 0.08 for no inhibitor and INDO, respectively), and both of these pD_2 values were greater (P = 0.01) than those for CAR arteries incubated with IBTX or NLA (7.17 and 7.16 vs. 4.97 and 5.50 ± 0.22 for no inhibitor, INDO, IBTX, and NLA, respectively).

Maternal MP did not impact the E_{max} of the BK dose response curve in CAR arteries with no inhibitor (P = 0.45; Figure 2A), with IBTX (P = 0.20; Figure 2B), with INDO (P = 0.09; Figure 2C), or with NLA (P = 0.42; Figure 2D). In MP60 ewes, the BK dose response E_{max} was similar (P = 0.79) between the curves for CAR arteries with no inhibitor and with CAR arteries incubated with INDO (Figure 3A), but the E_{max} was lower (P = 0.02) for CAR arteries with no inhibitor compared with arteries incubated with IBTX or NLA (Figure 3A). There were similar results in the MP100 ewes (Figure 3B), where CAR arteries with no inhibitor had a similar (P = 0.70) E_{max} to CAR arteries incubated with INDO, but had a lower (P = 0.01) E_{max} compared to CAR arteries incubated with IBTX or NLA (Figure 3B). The same held true in the MP140 ewes (Figure 3C). The CAR arteries with no inhibitor had similar a similar (P = 0.65) E_{max} to CAR arteries incubated with INDO, but had a lower (P = 0.01) E_{max} compared to CAR arteries incubated with IBTX or NLA (Figure 3C). Overall, the BK dose response E_{max} for CAR arteries with no incubator was similar (P = 0.79) to the E_{max} for CAR arteries incubated with INDO, but lower (P = 0.01) compared to CAR arteries incubated with IBTX or NLA (Figure 4A).

Cotyledonary artery bradykinin dose response curves in the absence or presence of an inhibitor. There were no interactions ($P \geq 0.92$) on the BK dose response curves in COT arteries with no inhibitor (Figure 5.5A), or for COT arteries incubated with IBTX (Figure 5.5B), INDO (Figure 5.5C), or INDO (Figure 5.5D). There was no treatment by dose by dose response curve interaction (P = 0.99), so the dose by dose response curve interaction, dose main effect, and dose response curve main effect were

analyzed for each treatment (MP60, MP100, and MP140) individually (Figure 5.6). There were no dose by dose response curve interactions ($P \geq 0.52$) on the BK dose response curves in MP60 (Figure 5.6A), MP100 (Figure 5.6B), or MP140 (Figure 5.6C) ewes.

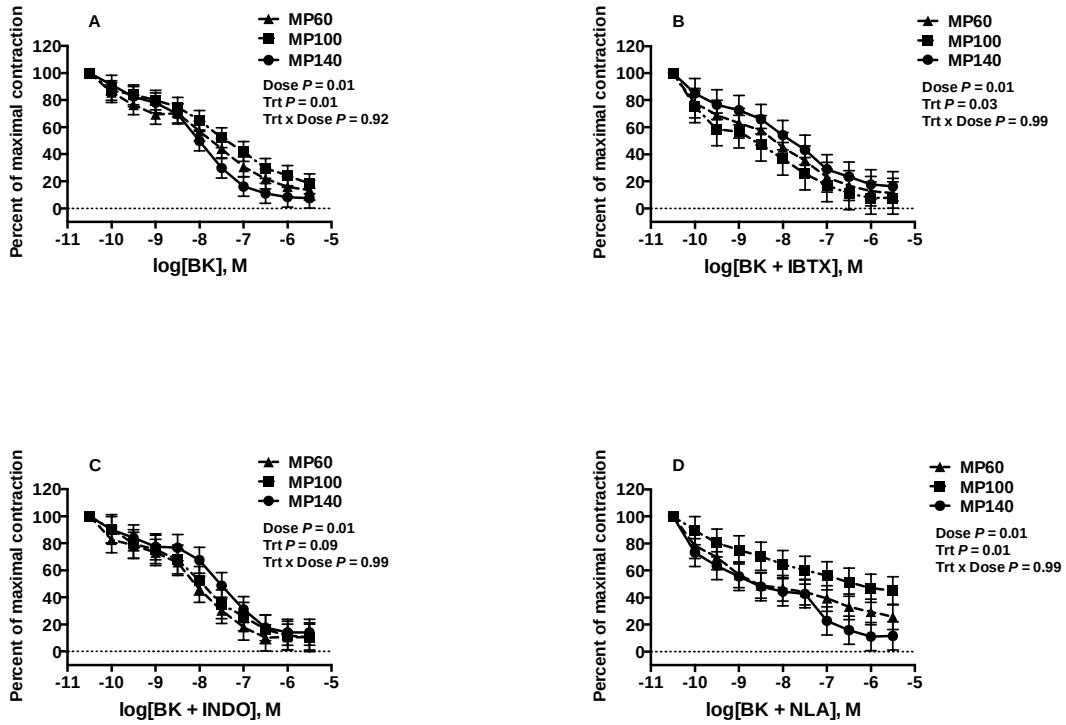

Figure 5.5. The interaction of maternal metabolizable protein (**MP**) level and bradykinin (**BK**) dose on the BK dose response curve in cotyledonary arteries (**A**) in the absence of an inhibitor (no inhibitor, **NI**), (**B**) after incubation with iberiotoxin (**IBTX**), (**C**) after incubation with indomethacin (**INDO**), and (**D**) after incubation with *N*(omega)-L-arginine methyl ester (**NLA**). Maternal diets were formulated to be isocaloric and provide 60% of MP requirements (**MP60**), 100% of MP requirements (**MP100**), or 140% of MP requirements (**MP140**). Diets were fed from day 100 to 130 of gestation.

Cotyledonary artery EC_{50}, pD_2, and E_{max} values. In COT arteries, the pD_2 value for the BK dose response curve was lower ($P = 0.01$) in MP100 ewes compared with MP60 and MP140 ewes, which were similar ($P = 0.13$; 7.36 vs. 7.81 and 8.05 ± 0.13 for MP100, MP60, and MP140, respectively). In COT arteries incubated with IBTX, the pD_2 value was higher $P = 0.01$) in MP100 ewes compared with MP140,

with MP60 being intermediate ($P \geq 0.11$; 8.67 vs. 7.83 ± 0.19 and 8.26 ± 0.19 for MP100, MP140, and MP60, respectively). In COT arteries incubated with INDO, MP60 ewes had a greater ($P = 0.01$) pD_2 value compared with MP140 ewes, with MP100 ewes being intermediate ($P \geq 0.11$; 8.15 vs. 7.60 ± 0.15 and 7.95 ± 0.16 for MP60, MP140, and MP100, respectively). In COT arteries incubated with NLA, MP100 ewes had a lower ($P = 0.01$) pD_2 value compared with MP60 and MP140 ewes, which were intermediate ($P = 0.09$; 6.37 vs. 7.90 and 8.42 ± 0.35 for MP100, MP60, and MP140, respectively). The BK dose response curves for COT arteries incubated with INDO and NLA had similar ($P \geq 0.27$) pD_2 values compared to COT arteries with no inhibitor (7.90 and 7.69 vs. 7.76 ± 0.14 for INDO, NLA, and no inhibitor, respectively). The pD_2 value for COT arteries with no inhibitor was lower ($P = 0.01$) compared with COT arteries incubated with IBTX (7.76 vs. 8.23 ± 0.10 for no inhibitor and IBTX, respectively). Maternal MP did not affect the E_{max} of the BK dose response curve in COT arteries with no inhibitor ($P = 0.61$; Figure 5.5A), with IBTX ($P = 0.84$; Figure 5.5B), with INDO ($P = 0.95$; Figure 5.5C), or with NLA ($P = 0.06$; Figure 5.5D). The E_{max} was similar ($P \geq 0.64$) for all dose response curves within the MP60 (Figure 5.6A) and MP140 ewes (Figure 5.6C), but in MP100 ewes, the E_{max} was greater ($P = 0.01$) in COT arteries incubated with NLA compared with COT arteries with no inhibitor (Figure 5.6B). Overall, the BK dose response E_{max} for COT arteries with no incubator was similar ($P = 0.11$) to the E_{max} for COT arteries incubated with IBTX, INDO, and NLA (Figure 5.4B).

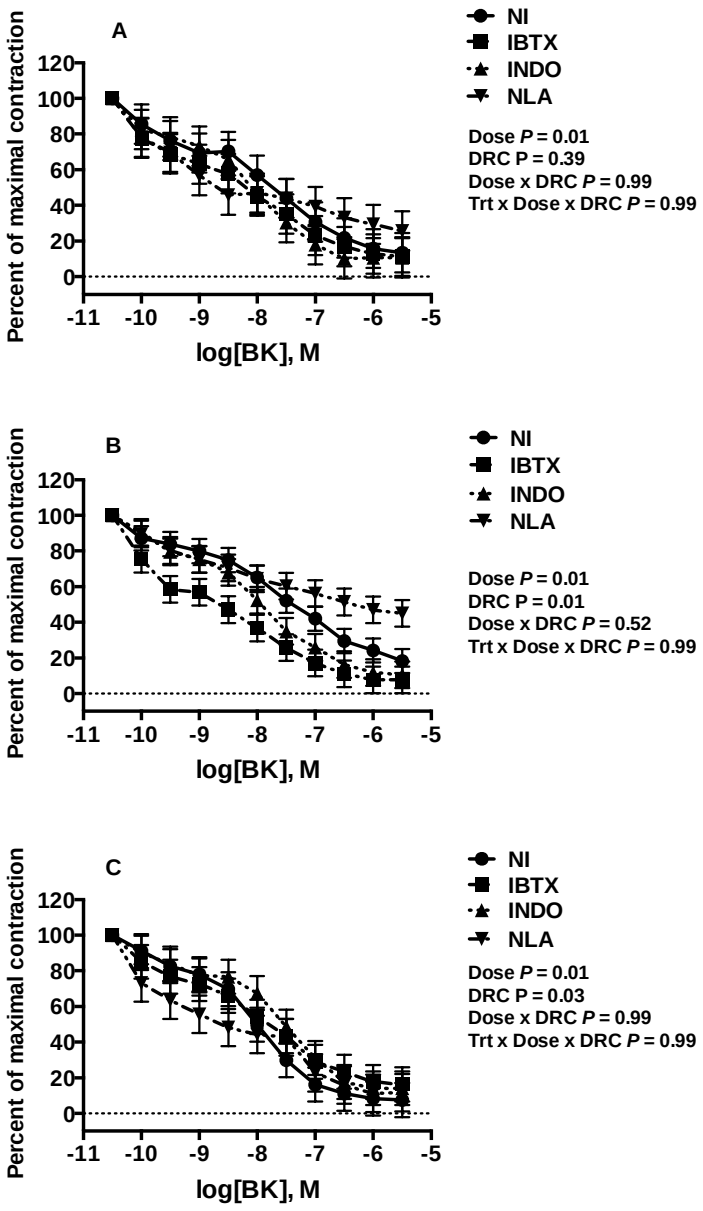

Figure 5.6. The effect of dose response curve (**DRC**) in cotyledonary (**COT**) arteries on the bradykinin (**BK**) DRC within each dietary treatment. Ewes were fed diets formulated to provide (**A**) 60% of metabolizable protein (**MP**) requirements (**MP60**), (**B**) 100% of MP requirements (**MP100**), and (**C**) 140% of MP requirements (**MP140**). The COT arteries were incubated with no inhibitor (**NI**) or incubated with iberiotoxin (**IBTX**), indomethacin (**INDO**), or *N*(omega)-L-arginine methyl ester (**NLA**). Diets were fed from day 100 to 130 of gestation. †NI is statistically different from IBTX, NLA, and INDO ($P \leq 0.05$). *****NI is statistically different from IBTX and NLA ($P \leq 0.05$). $^{\#}$NI is statistically different from INDO ($P \leq 0.05$).

Endothelial nitric oxide synthase, GUCY1B3, BKR1, and BKR2 mRNA expression

The mRNA expression data is expressed as a ratio of the gene of interest to 18s. Maternal metabolizable protein level did not significantly influence the mRNA expression of *eNOS* (P = 0.54; 1.32, 1.49, 1.42 ± 0.10 arbitrary units for MP60, MP80, and MP100, respectively), *GUCY1B3* (P = 0.47; 1.25, 1.34, 1.34 ± 0.06 arbitrary units for MP60, MP80, and MP100, respectively), *BKR1* (P = 0.28; 1.15, 1.34, 1.36 ± 0.10 arbitrary units for MP60, MP80, and MP100, respectively), or *BKR2* (P = 0.30; 1.32, 1.63, 1.44 ± 0.14 arbitrary units for MP60, MP80, and MP100, respectively) in the CAR arteries. Similarly, in the COT arteries, maternal metabolizable protein did not significantly influence the mRNA expression of *eNOS* (P = 0.93; 1.24, 1.26, 1.25 ± 0.04 arbitrary units for MP60, MP80, and MP100, respectively), *GUCY1B3* (P = 0.92; 1.22, 1.20, 1.23 ± 0.16 arbitrary units for MP60, MP80, and MP100, respectively), *BKR1* (P = 0.71; 1.10, 1.14, 1.13 ± 0.03 arbitrary units for MP60, MP80, and MP100, respectively), or *BKR2* (P = 0.26; 1.45, 1.34, 1.31 ± 0.06 arbitrary units for MP60, MP80, and MP100, respectively).

Discussion

This study aimed to investigate the effects of maternal metabolizable protein level on ewe body weight, fetal mass and size, placental characteristics, bradykinin-induced vasorelaxation of maternal and fetal placental arteries, the mechanisms of bradykinin-induced vasorelaxation in placental arteries, and the mRNA expression of key proteins and receptors in the placental arteries in near term ewes. This is an important study as no data exist on the role of maternal MP in bradykinin-induced vasodilation in maternal and fetal placental arteries or the mechanisms responsible for this vasodilation in sheep.

Limited data exist on the effects of maternal protein level and vascular function. Brawley et al. (2003) found that male offspring from rats fed a low protein diet during gestation had a decreased sensitivity to bradykinin in mesenteric arteries. Maternal protein restriction has also been shown to decrease sensitivity to acetylcholine, another endothelium-dependent vasodilator in the uterine artery of pregnant rats (Itoh et al., 2002), in the mesenteric arteries of virgin and pregnant rats (Koumentaki et al., 2002), in mesenteric arteries of pregnant female offspring (Torrens et al., 2002), and in mesenteric arteries of male offspring (Brawley et al., 2003). While the results from these rodent studies are interesting, it is difficult to compare the data to the current study. None of these studies examined the

effect of a maternal protein level on placental arteries, and some of the studies were measuring vascular responses in the offspring rather than the dam (Itoh et al., 2002; Torrens et al., 2002; Brawley et al., 2003). Further, while both acetylcholine and bradykinin elicit vasorelaxation via endothelium-dependent pathways, studying vascular responses to acetylcholine does not give any indication how these arteries would respond to bradykinin. Wirth et al. (1996) studied vascular responses to acetylcholine and bradykinin in spontaneously hypertensive rats and control rats and found that the hypertensive rats had impaired responses to bradykinin throughout life, but that impairments to acetylcholine-relaxation were only observed in elderly rats. Wirth et al. (1996) concluded that vascular dysfunction may be more closely related to impaired bradykinin-induced vasorelaxation than with acetylcholine-induced responses. This makes the current study very important as it, along with Lekatz (Chapter 3), are the first to study the effects of maternal protein level on bradykinin-induced vascular responses in placental arteries in sheep.

Lekatz (Chapter 3) found that despite no negative effects of restricting maternal MP on fetal or placental measurements, the vascular function of placental arteries were altered. Ewes restricted to 60 or 80% of MP from day 100 to 130 of gestation had fetal placental arteries that were more sensitive to BK-induced vasodilation. In the current study, there was an overall treatment effect on the BK dose response curve in fetal placental arteries, but when the means were separated, no significant differences existed. However, the pD_2 value of the BK dose response curve in fetal placental arteries was lower in ewes receiving 100% of MP requirements compared to ewes that were receiving either 60 or 140% of MP requirements. This means that there was a leftward shift of the BK dose response curve in the MP60 and MP140 ewes, which indicates these ewes had fetal placental vessels that were more sensitive to BK-induced vasodilation compared with ewes meeting MP requirements during late gestation.

Currently, the exact mechanism of bradykinin-induced vasorelaxation in placental arteries of sheep is unknown. Further, how maternal MP level might affect these mechanisms is also unknown. This novel study is important since understanding how bradykinin elicits vasorelaxation in placental arteries may lead to potential therapeutics for compromised pregnancies.

Bradykinin is an endothelium-dependent vasorelaxant. Endothelium-dependent vasorelaxants elicit vasodilation through one of three mechanisms including nitric oxide (NO; Ignarro et al., 1987; Furchgott, 1988; Moncada et al., 1991; Lamontagne et al., 1992), endothelium-derived hyperpolarizing

factor (EDHF; Beny and Brunet, 1988; Chen et al., 1988; Feletou and Vanhoutte, 1988; Tayler et al., 1988) and prostacyclin (PGI2; Lamontagne et al., 1992) pathways. In order to study which pathway is responsible for bradykinin-induced relaxation in maternal and fetal placental arteries in sheep, each of these pathways needs to be blocked before obtaining a bradykinin dose response curve. Iberiotoxin (IBTX) is a selective inhibitor of the high-conductance calcium-activated potassium channels and, therefore, was used to inhibit the EDHF pathway. Indomethacin, a cyclooxygenase inhibitor, was used to block the PGI2 pathway, and *N*(omega)-L-arginine methyl ester (NLA) an inhibitor of nitric oxide synthase (NOS) was used to block the NO pathway. The resulting BK dose response curves were compared to a BK dose response curve in the absence of an inhibitor. It appears that in maternal placental arteries, bradykinin-induced vasodilation is mediated by both EDHF and NO, but not by PGI_2. Interestingly, the data from the fetal placental arteries indicates that a novel pathway may be responsible for bradykinin-induced vasodilation in fetal placental arteries in sheep.

In the maternal placental arteries, there was no treatment by dose interaction on the vascular responses to the BK dose response curves in the presence of inhibitors (IBTX, INDO, and NLA). The pD_2 values of the BK dose response curve with no inhibitor (NI) and with INDO were similar. Further the maximal response to BK was similar between the NI and INDO dose response curves. The similar pD_2 and E_{max} values for NI and INDO indicate that indomethacin does not prevent bradykinin-induced vasorelaxation in maternal placental arteries, and perhaps bradykinin does not elicit vasorelaxation through the PGI2 pathway in maternal placental arteries.

Pregnant sheep have elevated levels of prostaglandins and a high venous-arterial concentration difference of prostaglandins exists across the gravid uterus of sheep (Mitchell et al., 1980). Further, the uterine vascular bed has been shown to produce various prostaglandins (Terragno, et al., 1974). Magness et al. (1985) demonstrated that the pregnant uterine artery of sheep is capable of producing PGI2. In addition, uterine artery endothelial cells from pregnant sheep have higher protein expression of cyclooxygenase-I and increased prostacyclin production compared with uterine artery endothelial cells from nonpregnant sheep (Jobe et al., 2013). Prostacyclin levels were not measured in the current study, but despite the possibility of increased PGI2 during late gestation, it does not appear that bradykinin induces vasorelaxation of the maternal placental arteries via release of prostacyclin. This is in

agreement with Chaudhuri et al. (1993) who investigated the role of both NO and PGI2 in maintaining low vascular tone of the umbilical artery and vein in humans. Results from the Chaudhuri et al. (1993) study indicate that NO is more important than PGI2 for maintaining low vascular tone of feto-placental arteries.

When maternal placental vessels were subjected to IBTX and NLA, the curves showed a rightward shift compared to the no inhibitor curve, which means that both IBTX and NLA did prevent bradykinin-induced vasorelaxation in maternal placental arteries, indicating that both EDHF and NO are important for bradykinin-induced vasorelaxation in maternal placental arteries in sheep.

Placental endothelial NO synthase specific activity and NO production is increased in pregnancy in sheep (Kwon et al., 2004; Vonnahme et al., 2005). Inhibition of NOS by NLA in late pregnant sheep decreases uterine venous NO second messenger cGMP levels (Rosenfeld et al., 1996) and uterine blood flow (Miller et al., 1999). Therefore, vasodilation during the last three months is partly mediated through the rise in NO production. It is not surprising that inhibiting NOS in near term sheep attenuates maternal placental arterial response to bradykinin.

In the fetal placental arteries, there was no treatment by dose interaction on the vascular responses to the BK dose response curves in the presence of inhibitors (IBTX, INDO, and NLA). Interestingly, none of the inhibitors used prevented vasorelaxation in the fetal placental arteries; all of the dose response curves had similar E_{max} values. The observed relaxation in the fetal placental arteries is likely due to an EDHF that is not inhibited by IBTX. Indeed, data regarding EDHF-type responses are continually growing, both in the number of studies and in complexity. These EDHF-type responses may be mediated by several factors or pathways depending on the type of vasculature, the species, and the physiological environment (reviewed in Luksha et al., 2009). Luksha et al. (2010) investigated the potential role of epoxyeicosatrienoic acids, which are metabolites of arachidonic acid through the CYP2C9 pathway, hydrogen peroxide, and communication between gap junctions as EDHF-type responses in human myometrial arteries. Results from the Luksha et al. (2010) study show that the myometrial arteries relaxed in the presence of NO and PGI2 inhibitors, but that the combination of NO, PGI2, and hydrogen peroxide inhibitors attenuated the bradykinin response. Similarly, inhibiting NO, PGI2, and gap junction communication (with 18-a-glycyrrhetinic acid) also decreased arterial response to bradykinin. Baragatti et al. (2007) observed relaxation of the mouse ductus arteriosus in response to

bradykinin despite inhibiting K+ channels, prostaglandins, and NO. This laboratory hypothesized that hydrogen sulfide might manifest itself once the classical active relaxants were inhibited (Baragatti et al., 2013). Indeed, only after inhibiting the ductus arteriosus with a cocktail to block prostaglandins, NO, K+ channels, and enzymes responsible for hydrogen sulfide production, was bradykinin-induced relaxation prevented (Baragatti et al., 2013). Hydrogen sulfide is a relatively newly described endothelium-dependent relaxing factor, but there is evidence to support its role in mediating vascular tone (reviewed in Wang, 2009). The data of Lukska et al. (2010) and Baragatti et al. (2007; 2013) indicate that there are endothelium-dependent relaxants other than the classical vasorelaxants (NO and PGI2) that are important for controlling vascular relaxation. In the current study, fetal placental arteries relaxed despite blocking K+ channels, cyclooxygenase 1 and 2, and nitric oxide synthase, which is likely due to an EDHF-type vasorelaxant. Further studies are needed to determine exactly what mechanism is responsible for this vasorelaxation in fetal placental arteries.

It is interesting to note that in the current study, the maternal and fetal placental arteries responded to bradykinin through different mechanisms. This is important when considering possible therapeutics for compromised pregnancies. Care should be taken to address what vascular beds are the target of such therapeutics, as the two vasculature systems are mediated by different mechanisms.

The mRNA expression of the BK receptors 1 and 2 (BKR1 and BKR2) was analyzed in the present study. Because one of the mechanisms of BK-induced relaxation involves nitric oxide, the mRNA of eNOS and it's receptor soluble guanylate cyclase was also analyzed. The mRNA expression of all genes investigated was similar among all treatments in both maternal and fetal placental arteries. This is similar to Lekatz (Chapter 3), where maternal MP restriction did alter mRNA expression of these genes. Lekatz (Chapter 3), observed an overall treatment effect on the maternal and fetal placental arterial response to BK. Because of those findings, one might expect to see differences among the three MP diets in mRNA expression of BK receptors 1 and 2 in these arteries; however, this was not the case. This is important because it stresses the importance of vascular function studies rather than focusing only on mRNA expression data.

Maternal MP did not impact ewe BW at slaughter or the percentage change from day 100 in ewe body weight at slaughter. Lekatz (Chapter 3) found that restricting MP to 60% of the requirements from

day 100 to 130 of gestation reduced the percentage change in BW at day 130 compared with ewes meeting 100% of MP requirements; however, ewes from both groups had similar BW at slaughter. In the current study, there was a treatment by day of gestation interaction for ewe BW throughout gestation; however, when the means were separated, substantial differences were not revealed ($P > 0.05$). Van Emon et al. (2013), fed similar diets to a larger group of ewes (n = 169) and found that at lambing, ewe BW increased linearly as MP in the diet increased. It is possible that a linear effect on ewe BW and the percentage change in ewe BW would have been observed in the current study if a larger sample size was used or if the ewes were fed the diets through gestation.

Maternal undernutrition has been shown to cause fetal growth restriction in sheep (Charlton and Johengen, 1985; Holst et al., 1986; Faichney and White, 1987; Holst et al., 1992; Bloomfield et al., 2000; Vonnahme et al., 2003; Scheaffer et al., 2004; Lekatz et al., 2010a, 2010b; Lemley et al., 2012). In the current study, maternal MP level did not alter fetal weight, fetal CCR, or fetal abdominal girth, which indicates that fetal mass and size is more dependent on the overall amount of calories received rather than the level of MP in the maternal diet. This is similar to Lekatz (Chapter 3), where MP was restricted to 60 or 80% of requirements during late gestation without impacting fetal growth measurements. When isocaloric diets that differed in the level of MP were fed to ewes from day 100 to lambing, there was no difference in lamb birth weight (Van Emon et al., 2013), which indicates that even if the MP treatments are fed through lambing, there are no negative effects on fetal weight. Also of importance, decreasing the MP level to 60% or increasing the MP to 140% of NRC requirements did not alter the percentage of assisted births or the percentage of lambs requiring treatment for illness at birth, which also indicates that decreasing MP level during gestation does not have negative effects on birthing or neonatal health and that increasing MP level does not have any additional benefit to birthing or neonatal health (Van Emon et al., 2013).

Maternal nutrient restriction in sheep has also been linked to altered placental weight in some studies (Charlton and Johengen, 1985; Faichney and White, 1987; McCrabb et al., 1991, 1992a, 1992b; Heasman et al, 1998; McMullen et al., 2005) but not in others (Vonnahme et al., 2003; Scheaffer et al., 2004; Holst et al., 1992; Bloomfield et al., 2000; Lemley et al., 2012; Lekatz et al., 2010a, 2010b). It is likely that the effects of maternal nutrition on placental characteristics vary among studies due to the

timing and severity of nutrient restriction. In the current study, maternal MP level from day 100 to 130 of gestation did not alter total placentome weight, placentome number, CAR weight, or COT weight. Further, fetal membrane weight, fetal fluid volume, and the fetal weight to placental weight ratio did not differ among treatments in the present study. This again indicates that not meeting or exceeding MP requirements during gestation does not impact placental measurements. The placental data from the current study mirrors the results from Lekatz (Chapter 3), where ewes were fed 60 or 80% of MP requirements without altering placental characteristics by day 130.

This novel study investigates the mechanisms of bradykinin-induced vasodilation in the maternal and fetal placental arteries in near term sheep. There are three important findings from this study. First, bradykinin-induced vasorelaxation in the maternal arteries appears to be partly due to both an EDHF involved with K+ channels and nitric oxide, but not a result of PGI2. Second, fetal placental arteries relaxed in the presence of inhibitors for K+ channels, nitric oxide synthase, and cyclooxygenase, which indicates there is an endothelium-dependent mechanism that has not yet been characterized in fetal placental arteries. Further studies are needed to investigate what this EDHF-type response might be. Finally, the maternal and fetal placental vasculature beds respond to bradykinin through different mechanisms. This becomes important when considering possible future therapeutics for compromised pregnancies.

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CHAPTER 6. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General conclusions

To my knowledge, very limited data exist on the effects of maternal metabolizable protein level on placental vascular function in the ewe. Because maternal nutrition during gestation has been linked to IUGR (Chandler et al., 1985; Barker et al., 1990; Barker et al., 1993; Wallace et al., 2001; Carr et al., 2012; Lemley et al., 2012), and because blood flow across the placenta is so important for normal fetal growth and development (Ford, 1995; Meschia, 1983; Redmer et al., 2004; Reynolds et al., 2005; Reynolds et al., 2006; Vonnahme et al., 2013), it is critical to understand how maternal protein levels might alter placental arteriole function.

These results indicate that while restricting or increasing maternal metabolizable protein during late gestation does not impair fetal or placental growth at day 130 of gestation, it may alter placental arterial function, particularly of the fetal placental arteries. Such adaptations to the placental cardiovascular system may play a part in programming of the fetal cardiovascular system and potential cardiovascular diseases later in life (Dahri et al., 1991; Langley et al., 1994; Ozaki et al., 2001; Itoh et al., 2002; Koumentaki et al., 2002; Torrens et al., 2002; Brawley et al., 2003).

Currently there are no good therapeutics for IUGR, and this is mainly due to a lack of knowledge on the mechanisms of IUGR. The results from this dissertation indicate that bradykinin-induced vasodilation of the maternal and fetal placental arteries is achieved through different pathways. This information is important when developing therapeutics for compromised pregnancies.

Overall, these studies indicate that maternal protein level during late gestation likely plays a key role in the function of the placental vascular system. Further, the maternal and fetal placental vascular beds should be viewed as two separate systems since the vasodilatory mechanisms of the arteries are likely mediated through different pathways.

Future directions

The data in these studies contribute to the small number of studies regarding the role of maternal metabolizable protein level during gestation on placental function in sheep; however, it is important to remember that vascular function measurements were obtained at one time point, day 130 of gestation. Future studies should be designed to gather vascular reactivity data in sheep at different time points

throughout gestation. Also, the protein studies presented in this dissertation focus on maternal diet during late gestation (day 100 to 130 of gestation). By this time, the placenta has reached its maximal size (Stegeman, 1974); therefore, studies investigating the effect of maternal protein level in sheep during early gestation may result in more dramatic vascular function alterations. For this reason, similar studies focusing on maternal protein level earlier in pregnancy are warranted.

Chapter 5 provides novel information on how bradykinin-induced vasodilation is mediated in the placental arteries. Given this information, future studies should be designed to better pinpoint the mechanism involved. For example, rather than incubating arterial rings with only one inhibitor, combinations of two or more inhibitors should be used to more exactly detect how big of a role each of the endothelium-dependent pathways has in placental vasorelaxation. Because the fetal placental arteries relaxed in the presence of all inhibitors tested, future studies are needed to determine what endothelial-derived hyperpolarizing like factor is mediating vasodilation of the fetal placental arteries. In addition, studies involving the use of bradykinin receptor 2 may be useful to insure that vasodilation is resulting from bradykinin binding to this receptor, as classically believed.

In summary, these studies contribute to the knowledge of maternal protein restriction during late gestation in ewes and provide information on the mechanisms regulating bradykinin-induced vasodilation in the ovine placenta arteries. The results are interesting and open the door for future studies including ones that are designed for multiple tissue collections. Another future step should include feeding different levels of metabolizable protein during different times of gestation, particularly earlier in gestation when placental development is rapid. Finally, additional studies to better understand the mechanisms bradykinin-induced vasodilation in the placental arteries are needed if future therapeutics for compromised pregnancies will target the placental vascular beds.

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