INFLUENCE OF MATERNAL NUTRIENT INTAKE ON PLACENTAL VASCULAR FUNCTION IN PREGNANT BEEF COWS

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Influence of maternal nutrient intake on placental vascular function in pregnant beef cows

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ABSTRACT

We hypothesized that global maternal nutrient restriction during early and mid-gestation followed by realimentation in pregnant beef cows would alter placental arterial vascular function. We tested changes in placental caruncular (**CAR**) and cotyledonary (**COT**) arterial sensitivity to bradykinin (**BK**), a potent vasodilator. Cows were randomly assigned to be nutrient restricted for 55 or 110 during early to mid pregnancy. On d 85, 140, and 254 cows were euthanized and CAR and COT arteries were isolated. Maternal nutrient restriction during early and mid-gestation allowed for placental compensation to overcome the loss of nutrients while realimentation returned placental arterial vosoactivity similar to control cows in response to BK. Further, CAR and COT placental arteries may respond to BK induced vasodilation through different pathways which is important when considering possible therapeutics for compromised pregnancies.

Key words: bradykinin, cow, placenta, vascular function

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LIST OF ABBREVIATIONS

BK ..... bradykinin

BK_{Ca} channels ..... large conductance Ca^{2+} activated K^{+} channels

BK1R ..... bradykinin 1 receptor

BK2R ..... bradykinin 2 receptor

°C ..... degrees Celsius

CAR ..... caruncular

C ..... cows received 100% nutrient requirements from d 30 - d 85 of gestation

CC ..... cows received 100% nutrient requirements from d 30 - d 140 of gestation

CCC ..... cows received100% nutrient requirements from d 30 - d 254 of gestation

COT ..... cotyledonary

CRC ..... concentration response curve

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

eNOS ..... endothelial nitric oxide synthase

EDHF(s) ..... endothelium derived hyperpolarizing factors

HK ..... high molecular weight kininogen

HOE 140 ..... icatibant acetate

IbTX ..... iberiotoxin

INDO.....indomethacin

IUGR.....intrauterine growth restriction

KCl..... potassium chloride

KKS.....kallikrein kinin system

KD ..... kallidin

LK .....low molecular weight kininogen

MP.....metabolizable protein

NE ..... norepinephrine

NI .....no inhibitor

NLA ..... *N*(nitro)-L-arginine

NRC ..... National Research Council

NO ..... nitric oxide

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

PSS .....physiological saline solution

PGI_2..... prostacyclin

R..... cows received 60% nutrient requirements from d 30 - d 85 of gestation

RC .....cows received 60% nutrient requirements from d 30 - d 85 with realimentation to 100% on d 85 until d 140 of gestation

RCC.....cows received 60% nutrient requirements from d 30 - d 85 with realimentation to 100% on d 85 until d 254 of gestation

RR ..... cows received 60% nutrient requirements from d 30 - d 140 of gestation

RRC.....cows received 60% nutrient requirements from d 30 - d 140 with realimentation to 100% on d 140 until d 254 of gestation

SEM ..... standard error of the mean

sGC .....soluble guanylate cyclase

TK .....tissue kallikrein

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

CHAPTER 1. LITERATURE REVIEW

Placental Function

The placenta is a specialized and unique organ formed during pregnancy that carries on itself the burden of a successful pregnancy. From the time of conception until parturition, the placenta acts as the central organ that ensures optimum fetal growth and development. The establishment of maternal-fetal interactions during the earliest stages of embryonic development is of monumental importance because the two grow together, entwined in order to sustain and replenish life in the womb.

Several different types of mammalian placentas have been identified based on either their shape (Hamilton, Boyd and Mossman, 1972) and/or composition of their fetal membranes (Flexner and Gellhorn, 1943). Humans, have a highly invasive placenta classified as discoid and hemochorial, in which all maternal tissue has disappeared and maternal blood bathes the trophoblast (Thomas, 1997) whereas; a ruminant placenta is non-invasive and characterized as cotyledonary and epitheliochorial, in which all three maternal layers are intact (Senger, 2005). Regardless of the mammalian placental anatomy, all types ensure optimum health of the offspring.

The primary function of the placenta is to regulate the fetal metabolic demands throughout gestation (Needham, 1934; Ramsey, 1982; Faber and Thornburg, 1983; Morris and Boyd, 1998; Reynolds and Redmer, 1995). In the cow, transplacental exchange occurs through specialized structures called the placentomes which forms an interface between the maternal and fetal systems. These can be visualized on the surface of uterine horns as convex, ovoid structures (King et al., 1979; Laven and Peters, 2001) ranging anywhere from 70-120 in number (Schlafer

et al., 2000), 2-3 cm thick and 10-12 cm long (Schlafer et al., 2000). Thus, the primary function of the placenta is to allow for the physiological exchange of respiratory gases, nutrients and elimination of fetal waste between the dam and the fetus (Meschia, 1983; Cross, 1998; Bell et al., 1999; Reynolds and Redmer 1995, 2001). The placenta also functions to provide an immunological barrier against various pathogens and/or the maternal immune system, thereby establishing the uterus as an immunologically privileged site; hence, preventing the rejection of the conceptus (Soares, 1993; Cross, 1998; Bell et al., 1999; Bainbridge, 2000; Walker et al., 2010). Additionally, the placenta also serves as an endocrine organ that secretes several hormones such as progesterone, estrogens (Hoffmann and Schuler, 2002), prolactin, and various growth factors (Anthony et al., 1995; Soares et al., 1998; Schlafer et al., 2000; Forsyth and Wallis, 2002; Gluckman and Pinal, 2003). These factors collectively, are crucial for establishing, nourishing and maintaining a viable and healthy fetus (Conley and Mason, 1990; Ogren and Talamantes, 1994; Solomon, 1994; Anthony et al., 1995).

Besides the above mentioned functions, the placenta also plays a pivotal role in developmental programming. Any insults to the maternal system *in utero* have the capacity of altering bodily systems and processes including the placenta and placental function thus, producing life-long alterations in the postnatal life (Godfrey and Barker, 2000). This is especially relevant in intrauterine growth restriction (**IUGR**) which is often caused by maternal under-nutrition during gestation that can lead to stunted placental and fetal growth and development (Barker, 1997). In fact, IUGR placentas in humans often display altered vasculogenesis (Krebs et al., 1996) and aberrant enzyme and hormone production (McMullen et al., 2005).

As previously described, the placenta aids in nutrient transfer which is primarily achieved by regulating the utero-placental blood flow (Reynolds and Redmer, 1995) to the fetus.

Therefore, an abnormal placentation caused as the result of IUGR leads to a decrease in utero-placental blood flow (Bell et al., 1999) resulting in fetal (Bell et al., 1999) and trophoblast (Myatt, 2006) hypoxemia, fetal hypoglycaemia, and pre-eclampsia (Myatt, 2006). In support of this concept, in a reduced utero-placental blood flow rodent model, Anderson et al. (2005, 2006) demonstrated a decrease in litter size, placental, and individual fetal weights. Moreover, the subsequent generations were programmed to exhibit persistent hypertension in their postnatal life.

While it is evident that IUGR poses a significant threat to fetal growth and development, an optimal treatment remains problematic. Therefore, future studies investigating the effects of IUGR on placental function and placental vasculature are highly important.

Placental Development

Early Gestation

Early gestation is a critical time for placental growth and development as it lays the foundation for the production of a viable and healthy offspring. The placenta begins to grow rapidly in order to support an exponential increase in fetal growth during late in gestation.

Upon fertilization, on d 4 the bovine embryo enters the uterus and undergoes a series of cell division forming a morula by d 5 and a blastocyst consisting of a fluid filled cavity called the blastocoele by d 7 (Betteridge and Flechon, 1988). The outermost single layer of cells surrounding the blastocoele forms the trophoblast which gives rise to the bulk of the placenta (Schlafer et al., 2000).

In the cattle, the placenta begins to form around d 18 to 19 of gestation when the trophoblast forms an intimate association with the uterine endometrium (Wooding and Morgan,

1993; Thie et al., 1998; Wathes and Wooding, 1980; MacLaren and Wildeman, 1995). This early attachment phase is a gradual process that occurs over several weeks (Melton et al., 1951) followed by the fusion of the trophoblast with the mesoderm to form the chorion.

The blastocyst continues to grow accumulating more fluid in the coelom that pushes the specialized group of cells called the inner cell mass at one end of the cavity. This inner mass of cells gives rise to the endoderm that lines the yolk sac and eventually forms the fetus. Alongside endoderm, the mesoderm also vascularizes and extends further around the blastocyst. The embryo continues to grow and elongate and is soon surrounded by a fluid filled cavity called an amnion (Schlafer et al., 2000). By d 20 of gestation, a sac-like structure called an allantois (Betteridge and Flechon, 1988) extends from the hindgut of a fetus into the mesoderm. An allantois possesses blood vessels required for vascularization and together with the chorion it gives rise to the "chorioallantoic placenta" (Mossman, 1987; Schlafer et al., 2000; Wooding and Burton, 2008).

In the bovine, before the establishment of maternal-fetal circulations, the fetus derives its energy from the endometrial histotroph (Melton et al., 1951; Stice et al., 1996; Thompson, 1997) which is soon replaced by specialized structures called the placentomes, which form an interface between the maternal and the fetal systems. Placentome development begins when the chorioallanotic membrane forms an intimate association with aglandular caruncular regions on a uterine wall. The placental membrane also contacts a uterine caruncle via finger like projections emerging from the chorion called the chorionic villi (Amoroso, 1952; Mossman, 1987), ultimately developing a cotyledon (Melton et al., 1951; Stice et al., 1996; Thompson, 1997). By d 30 of gestation, the caruncular (CAR) and the cotyledonary (COT) unit forms an intimate association giving rise to a placentome (Schlafer et al., 2000). Placentomes can be visualized as

slightly raised structures on the uterine surface by d 33 of gestation (King et al., 1979). On an average, 70 to 120 placentomes (Schlafer et al., 2000) could be found arranged in two dorsal and two ventral rows running lengthwise along the uterine horns. Placentomes function as the primary area of physiological exchange and enable the transfer of respiratory gases, nutrients and elimination of fetal waste between the dam and the fetus (Reynolds and Redmer, 1995, 2001).

By d 42 until d 67 of gestation, the placental villous tree branches out extensively (King et al., 1979) to increase the surface area for transplacental exchange (Perry et al., 1999). This exchange occurs through the uterine and umbilical vascular beds which are responsible for the allocation of blood flow to the CAR and the COT portion of the placenta (Ramsey, 1982; Mossman, 1987).

Mid-gestation

Various animal models have demonstrated placental function as a characteristic of fetal weight, placental size and vascularity, and uterine and umbilical blood flows (Adair and Thelander, 1925; Ibsen, 1928; Warwick, 1928; Hammond, 1935; Mckeown and Record, 1953; Alexander, 1964; Oh et al., 1975; Wotton et al., 1977; Christenson and Prior, 1978; McDonald et al., 1979; Prior and Laster, 1979; Hard and Anderson, 1982; Myers et al., 1982; Caton, 1984; Ford et al., 1984; Reynolds et al., 1984; Johnson et al., 1985; Metcalfe et al., 1998; Ferrell, 1989; Ferrell and Reynolds, 1992; Vonnahme et al., 2001; Di Naro et al., 2003). Additionally, the fetal growth trajectory could also be affected by the fetal and maternal genotype, epigenetic, and environmental factors including nutritional stress and various diseases (Redmer et al., 2004). Needless to say, any alterations in these factors during gestation would have a direct impact on the placental function thus, influencing nutrient uptake by the gravid uterus (Bell and Ehrhardt, 2002; Redmer et al., 2004).

Several mammalian studies have reported an exponential increase in the placental (Ferrell, 1989; Reynolds and Redmer, 1995; Bell et al., 1999) and fetal (Evans and Sack, 1973; Reynolds and Redmer, 1995) weights throughout gestation. In the ovine, maximum placental growth occurs early in gestation resulting in an increase in placentome weight by d 90 of gestation (Alexander, 1964; Stegeman, 1974). Following this, the placenta ceases to grow and the total placentome weight decreases by late gestation (Barcroft and Barron, 1946), despite a positive correlation between placental and fetal weight (Alexander, 1964).

In contrast with the ovine placental growth, in cattle and humans, the increase in placental and fetal weights occurs at different time points during gestation such that the overall increase in placental weight is far less than that of the absolute fetal weight (Ferrell et al., 1976; Ferrell and Ford, 1980; Reynolds et al., 1990; Reynolds and Redmer, 1995). This is due to the fact that maximal placental growth and differentiation occur during the first half of gestation (Ferrell, 1989; Reynolds and Redmer, 1995; Bell et al., 1999) with only limited growth in the last half to promote the dramatic fetal growth in the last trimester of gestation (Ibsen, 1928; Warwick, 1928; Hammond, 1935; Barcroft and Barron, 1946; Metcalfe et al., 1988; Ferrell, 1989; Reynolds and Redmer, 1995). This is also accentuated by the fact that in the cow, from d 100 to d 250 of gestation the CAR weight increased a little more than 2 fold although, the DNA concentration remained constant when compared with the COT. On the contrary, the COT DNA increased by 3 fold during the last two-thirds of gestation, indicative of COT hyperplasia occurring throughout gestation to keep pace with the ever increasing metabolic demands of the fetus (Reynolds et al., 1990; Reynolds and Redmer, 1995). Thus, the placenta undergoes rapid tissue remodelling from mid to late gestation which influences placental function and has the

capacity of altering blood flow to the CAR and COT sides of the placenta (Reynolds and Redmer, 1995; Reynolds et al., 2006).

Late Gestation

As described earlier, in ruminants, the first half of gestation marks maximal growth and differentiation of the placenta which is achieved by rapid proliferation of blood vessels within the placentome (Reynolds et al., 1992). The placental weight increases exponentially throughout gestation in the bovine, however the rate of increase slows significantly compared with the fetal weight during late gestation (Ferrell et al., 1976; Ferrell and Ford, 1980; Ferrell, 1989; Reynolds et al., 1990; Reynolds and Redmer, 1995); whereas, the placental mass ceases in the ovine (Barcroft, 1946; Wallace, 1948; Alexander, 1964). By late gestation, the primary function of the placenta is to support the exponential growth of the fetus during the last half of gestation (Ferrell, 1989; Reynolds and Redmer, 1995; Metcalfe, 1998). This is not only achieved by an exponential increase in the uterine and umbilical blood flows (Ferrell and Ford, 1980; Morriss et al., 1980; Vorherr, 1982; Ford et al., 1984; Reynolds et al., 1985) but also, a uniform increase in the allocated blood flow to the CAR and COT placental vascular beds from mid to late gestation (Makowski et al., 1968; Rosenfeld et al., 1974; Meschia, 1983).

By late gestation, in ewes and cattle, the magnitude of uterine blood flow is enhanced by 3-20% (Rosenfeld et al., 1974; Reynolds et al., 1986; Reynolds and Redmer, 1995; Panarace et al., 2006) with 82% being exclusively directed to the caruncles in the ewes (Rosenfeld et al., 1974; Rosenfeld and Fixler, 1977). Additionally, most of the increase in the gravid uterine blood flow is accompanied by a large decrease in uterine vascular resistance and an increase in arterial diameter due to vasodilation (Rosenfeld, 1984; Ford, 1995; Magness, 1998; Magness et al., 2005). Similarly, there is an exponential rise in the umbilical blood flow in both, the bovine and

the ovine species, which keeps pace with the fetal growth (Rudolph and Hymann, 1970; Reynolds and Ferrell, 1987) and helps support 75-85% of the total fetal growth during the last 0.4 months of gestation (Robinson et al., 1977; Prior and Laster, 1979).

Therefore, an understanding of factors that impact the placental vascular development and function with relation to utero-placental blood flow would influence the nutritional and physiological status of the offspring both, *in utero* and in postnatal life.

Developmental Programming and Influence of Maternal Nutrient Intake on Placental Vascular Development in Ruminants

The concept of developmental programming explains that any insults or stressors to the maternal system while the fetus is *in utero* or during the neonatal period have the capacity of altering bodily systems and processes thus, producing life-long alterations in the postnatal life (Godfrey and Barker, 2000). Maternal suboptimal nutrition is one such stressor during pregnancy that is not only associated with a high risk of neonatal mortality and morbidity but can also program metabolic and reproductive changes in the offspring (Wallace, 1948; Wallace et al., 1999a; Godfrey and Barker, 2000). In fact, livestock in the United States are often under a poor nutritional environment during pregnancy (Wu et al., 2006). This is mainly due to poor pasture conditions or an early breeding of peripubertal dams; thus, initiating a competition for nutrients between maternal body growth and fetal growth.

There is increasing evidence that maternal under-nutrition during gestation in humans and livestock causes IUGR which can lead to stunted placental-fetal growth and development (Barker and Clark, 1997; Bell and Ehrhardt, 2002). In the United States, IUGR affects ~5% of the total human infants (Marsal, 2002). The incidence of IUGR resulting in low birth weight

infants ranges from 7-8% in the United States (NLM, 2002a, b; NVSR, 2004); 6% in Europe and the Western Pacific, and 24% in Southeast Asia (World Health Statistics, 2010). In fact, in livestock species including the cattle, nutritionally induced IUGR affects fetal nutritional and physiological status, negatively influencing animal's body composition and reproductive performance in postnatal life (Wu et al., 2006; Funston et al., 2010). Additionally, the compromised fetal growth makes the offspring more susceptible to metabolic disorders, obesity, hypertension, insulin resistance, organ dysfunction, and cardiovascular diseases in their later life (Barker et al., 1993; Godfrey and Barker, 2000; Barker, 2004; Wu et al., 2006; Ford et al., 2007; Vonnahme et al., 2007; Dong, 2008; Funston et al., 2010). While, it is evident that IUGR poses a significant threat to the fetal growth and development, designing potential therapeutic strategies to overcome IUGR remains problematic.

As described previously, maximal placental growth occurs during the first half of gestation, followed by the fetal growth during the last half of gestation (Ferrell et al., 1976; Ferrell and Ford, 1980; Reynolds et al., 1990; Reynolds and Redmer, 1995). This makes the fetal growth trajectory highly sensitive to the timing, duration, and severity of the maternal nutritional insult (Robinson et al., 1999). While there are quite a few studies elucidating the role of maternal suboptimum nutrition during pregnancy in humans and livestock (Meschia, 1983; Magness, 1998; Konje et al., 2003; Reynolds et al., 2006; Vonnahme et al., 2007; Wallace et al., 2008); studies investigating the effects of maternal under-nutrition followed by realimentation on the bovine placental vasculature are limited. Further, the precise mechanisms mediating the effects of maternal nutrient restriction and/or realimentation on the bovine placental function and particularly on the CAR and COT arterial vasculature have not been revealed. Therefore, the

purpose of our research is to provide a better understanding of the bovine placental vascular remodelling during gestation which regulates the fetal growth and development.

Early Gestation

Maternal nutrient requirements might appear trivial during early gestation but, it plays a significant role in the placental-fetal development since, the earliest stages of embryonic development (Bishonga et al., 1996; Sugden, 2002; Waterland, 2004). Studies in ewes and rodents suggest that poor maternal nutrition at the time of conception leads to reduced number of cells in the inner cell mass (Robinson et al., 1994; Walker et al., 1996) which not only results in retarded fetal growth and organ function but also programs the offspring to exhibit hypertension in postnatal life (Kwong et al., 2000; Barker, 2001). Epidemiologic evidence in humans (Ravelli et al., 1988; Barker et al., 1993; Godfrey et al., 1996; Godfrey and Barker, 2000) and ewes (Whorwood et al., 2001) show that mothers who were nutritionally challenged during the first half of gestation were unable to supply nutrients to satisfy the fetal demands and hence, produced newborns that were proportionally longer and thinner than normal.

Maternal under-nutrition has a significant impact on the placental growth in humans and ruminants. According to the study conducted by Lao and Wong (1996), the ratio of placental to birth weight was higher in babies whose mothers were lacking in adequate oxygen supply or nutrient resulting in placental enlargement at birth. Lumey (1998) also reported that undernourished human mothers during the first trimester delivered heavier and enlarged placentas at term. While, an enlarged placenta might be an adaptive response to compensate for the loss of nutrients during early gestation; there is increasing evidence that this catch up growth is in fact detrimental to the offspring making them more susceptible to metabolic disorders,

obesity, hypertension, insulin resistance, dislipidemia, organ dysfunction, and cardiovascular diseases in later life (Barker et al., 1993; Godfrey and Barker, 2000; Barker, 2004; Wu et al., 2006; Ford et al., 2007; Vonnahme et al., 2007; Dong et al., 2008). Additionally, poor maternal nutrition during gestation has a capacity of producing epigenetic alterations, such that its adverse effects are not confined to one generation but, are tagged along with the subsequent generation to follow (Anderson et al., 2006; Ismail-Beigi et al., 2006; Wu et al., 2006).

Similar to humans, compensatory placental growth was also observed in various models of undernourished ewes from early to mid-gestation (Owens et al., 1986; McCrabb et al., 1986, 1991; Faichney and White, 1987). While, there are quite a few studies examining the role of maternal diet on fetal (Charlton and Johengen, 1985; Holst et al., 1986, 1992; Faichney and White, 1987; Bloomfield et al., 2000; Vonnahme et al., 2003; Scheaffer et al., 2004; Lemley et al., 2012) and placental (Charlton and Johengen, 1985; Faichney and White, 1987; McCrabb et al., 1991, 1992a, 1992b; Heasman et al, 1998; McMullen et al., 2005; Lekatz et al., 2010a, 2010b; Lemley et al., 2012) growth in the ewes; Perry et al. (1999) have investigated the effects of restricting a particular dietary component on placental function in beef cattle. They reported an increase in the dry cotyledon weight at parturition in heifers who were fed a low protein diet during early (d 0 to d 99) gestation. Their finding supports the idea of placental-fetal adaptations occurring in early gestation to support the fetal nutritional demands in late gestation.

Another study by Sullivan et al. (2009) examined the effects of maternal crude protein on the placental weights during early (d 1 to d 93) to mid gestation (d 93 to d 180) in beef heifers. A specific breed of beef heifers receiving low dietary protein (75%) during early gestation had an increase in placental weight compared with the high protein (250%) receiving group. However these findings were highly influenced by the maternal genotype as a decrease in placental weight

was observed when the same low protein concentration (75%) diet was fed to a different breed of beef heifers compared with the controls. Furthermore, no differences were observed in COT numbers, COT weight, COT surface area, and placental efficiency.

Comparable studies conducted by Vonnahme et al. (2007) and Zhu et al. (2007) investigated the effects of 50% global maternal nutrient restriction early in gestation (d 30 to d 125) on the bovine placental vasculature. Vonnahme et al. (2007) observed an increase in the placental angiogenic factors while no differences were reported in the capillary vascularity of the CAR and COT tissues. Further, the fetal and placentome weights were also reduced at the end of nutrient restriction period, on d 125 of gestation. On the contrary, Zhu et al. (2007) did not notice any differences in the fetal weight although; the placentome weight was reduced in the nutrient restricted cows when compared with the control cows. An increase in the COT arteriolar vascularity was also observed which perhaps enhanced the placental efficiency in the nutrient restricted cows (Zhu et al., 2007). The authors interpreted that these variations arose as a result of the distinct blood vessel types (capillaries vs arterioles) measured in the two studies.

While there are slight differences in the results, these studies strongly indicate that alterations in placental function might be a result of maternal under-nutrition during early gestation. Therefore, future studies elucidating the role of maternal under-nutrition during early gestation on placental function in relation with the placental vascular architecture within the cattle are imperative.

Mid-gestation

Several mammalian studies have reported an exponential increase in the placental (Ferrell, 1989; Reynolds and Redmer, 1995; Bell et al., 1999) and fetal (Evans and Sack, 1973;

Reynolds and Redmer, 1995) weights throughout gestation. As described previously, in the sheep, maximum placental growth occurs early until d 90 of gestation (Alexander, 1964; Stegeman, 1974), thereafter the placenta ceases to grow in mass (Barcroft and Barron, 1946) despite a positive correlation between placental and fetal weight (Alexander, 1964). Unlike in the ovine species, mid-gestation in the bovine is marked by maximal placental growth and differentiation (Barcroft and Barron, 1946; Metcalfe et al., 1988; Ferrell, 1989; Reynolds and Redmer, 1995; Bell et al., 1999) which continues to keep pace with increasing fetal demands throughout gestation.

Maternal nutrition during this crucial period of placental establishment has a significant impact on the fetal growth (Wallace, 1948; Wallace et al., 1999a; Godfrey and Barker, 2000). Numerous animal models investigating the effects of maternal diet in the ewes and cows have obtained variable results on the placental and fetal growth during mid-gestation in both species.

Clarke et al. (1998) reported a lower placental weight in ewes fed 50-60% of energy requirements compared with controls from d 30 to d 80 in gestation. Vonnahme et al. (2003) observed a marked reduction in fetal weight on d 78 of gestation when ewes were restricted to 50% of their dietary intake requirements compared with the control ewes receiving 100% of their energy requirements from d 28 to d 80 of gestation. A similar study conducted by Redmer et al. (2004) also demonstrated lower fetal weights and increased placental vascularity with no alterations in the placentome weight in ewes fed 60% global nutrient intake compared with control ewes fed 100% of energy requirements from d 50 to d 130 of gestation.

In the bovine, Perry et al. (1999) demonstrated that a period of protein restriction during early gestation (d 0 to d 99) followed by a high protein diet in mid-gestation (d 100 to d 198 of

gestation) led to enhanced trophectoderm volume density and enlarged placentas at term due to the greater development of microvilli. These researchers suggested that larger placentas would create an optimal design for absorption of nutrients to help support the fetal growth during late gestation.

Looking particularly at the realimentation studies in the bovine, documented responses by Vonnahme et al. (2007) and Zhu et al. (2007) show that a period of nutrient restriction (d 30 to d 125 of gestation) followed by realimentation (d 125 to d 250 of gestation) produce contrasting results in the CAR and COT placental tissues. Upon realimentation (d 125 to d 250 of gestation), in the under-nourished cows, the COT capillary area density, COT capillary surface density and COT capillary number density was markedly reduced whereas, the CAR capillary surface density was increased compared with the control cows. Additionally, the total placentome weight and the COT weight continued to be lower throughout gestation, even after realimentation (Zhu et al., 2007).

On the contrary, in the ewe, placental growth was enhanced upon realimentation in ewes when preceded by a period of nutrient restriction from early to mid-gestation (Foote et al., 1958; Robinson et al., 1995; Heasman et al., 1999; McMullen et al., 2005). A similar study by DeBarro et al. (1992) yielded similar results where placental hypertrophy was enhanced in well-nourished ewes subjected to nutrient restriction during mid-gestation; however, a period of poor nutrition during mid-gestation in formerly undernourished ewes produced detrimental effects on placental and fetal growth. These findings led the researchers to believe that the placental compensation might be directly related with the maternal nutrient store at the time of conception (McCrabb et al., 1992; Robinson et al., 1994).

Overall these studies indicate that maternal nutrition during mid-pregnancy significantly influences the vascular function of the placenta which has the capacity of altering fetal growth. There are only a few studies examining the role of realimentation on the placental vascularity in the cow hence, any conclusions are tentative and future studies are warranted. Furthermore, the differences in the placental growth and vascularity in the ovine and the bovine species is quite evident; thus, it is imperative that the two species are studied separately and compared with caution.

Late Gestation

By late gestation, the primary function of the placenta is to support the exponential growth of the fetus during the last half of gestation (Metcalfe, 1988; Ferrell, 1989; Reynolds and Redmer, 1995). Several models of underfed ewes during late gestation have found to hinder the fetal growth trajectory (Mellor, 1983; Robinson, 1983; Vincent et al., 1985; Parr et al., 1986) and transplacental exchanges. Nutrient restricted underfed adolescent (Luther et al., 2005) ewes experienced a 17% reduction in fetal weight and a 20% reduction in capillary area density. Likewise, adult ewes that underwent severe nutrient restriction during late gestation (d 120 to d 144 of gestation) had a marked decrease in fetal weight (17%) and uterine blood flow (20-33%) (Chandler et al., 1985; Leury et al., 1990; Newnham et al., 1991; Kelly, 1992; Arnold et al., 2001).

In humans, the Dutch Hunger Winter is a classic example of placental remodelling occurring as a result of maternal under-nutrition during different stages in gestation. A period of maternal nutrient restriction during early gestation resulted in compensatory placental growth; however, the same during late gestation resulted in lighter placentas and low birth weight infants

at parturition (Lumey et al., 1998). As previously described, the placenta aids in nutrient transfer which is primarily achieved by regulating the utero-placental blood flow (Reynolds and Redmer, 1995) to the fetus. An abnormal placentation in humans resulting from maternal under-nutrition during the last trimester causes IUGR leading to a decrease in utero-placental blood flow, fetal hypoxaemia, fetal hypoglycaemia, and organ dysfunction (Pardi et al., 1993; Marconi et al., 1996; Ferrazzi et al., 2000).

Langley-Evans et al. (1996a) constructed a rodent model to investigate the effects of protein restriction during gestation on fetal and placental development. Rats were either control fed receiving 14% casein or protein restricted receiving only 9% casein in their diet. These researchers observed an increase in placental (d 14 and d 20 of gestation) and fetal weight (d 14, d 18 and d 20 of gestation) in the restricted fetuses; however fetal weight and organ growth were significantly reduced at term (d 22 of gestation) indicating a difference in the restricted and the control fetal growth rate patterns during the last two days of gestation. Additionally, the restricted pups by week four were programmed to exhibit hypertension in their postnatal life.

Desai et al. (2005), Belkacemi et al. (2009), and Karadag et al. (2009) choose to study the impact of 50% nutrient restriction during late gestation in rats. A restricted nutrient supply led to a decrease in placental (Belkacemi et al., 2009) and fetal (Desai et al., 2005; Belkacemi et al., 2009; Karadag et al., 2009) weights. Furthermore, the researchers also noted an increase in the incidence of placental apoptosis at the site of placental labyrinth in the under fed rats (Belkacemi et al., 2009). Akohas et al. (1984) also showed a 17% and 26% reduction in mean fetal and placental weight in rats receiving 50% of energy requirements from d 5 to d 21 in gestation, compared with ad libitum fed control rats. Moreover, the placental insufficiency in the nutrient

restricted rats was due to increased vascular resistance leading to a 30% reduction (Akohas et al., 1983) in transplacental exchange.

Studies investigating the impacts of maternal under-nutrition and particularly realimentation on the bovine placental vasculature are quite limited. As mentioned previously, Vonnahme et al. (2007) observed a marked decrease in COT capillary area density, COT capillary surface density, and COT capillary number density upon realimentation in undernourished cows from d 125 to d 250 of gestation compared with the control cows. However, Zhu et al. (2007) observed no such differences in the COT arterioles measured. Vonnahme et al. (2007) and Zhu et al. (2007) both reported an increase in the CAR capillary surface density and CAR arteriolar density in the restricted cows than the control cows. The researchers interpreted, that the observed increase in the CAR tissue led to enhanced placental efficiency in the nutrient restricted cows compared with the control cows.

Overall, these studies strongly suggest that the fetal growth and development which maximally occurs in the last trimester of pregnancy is highly sensitive to the maternal stores of energy and any detrimental effects of maternal nutrition would lead to stunted placental-fetal development in any species. Furthermore, there is a lack of literature focusing on the impacts of maternal diet particularly on the bovine placental vasculature. Therefore, more studies are imperative.

Regulators of Blood Flow and Placental Vascular Function

Pregnancy in all mammals is energetically costly and marks a 20-50% increase in the maternal metabolic rate (Brody, 1938; Ferrell et al., 1976; Ferrell and Jenkins, 1985; Robson et al., 1989; Stock and Metcalfe, 1994). A large increase in the metabolic rate primarily supports

the dramatic alterations in the maternal cardiovascular system and the utero-placental vascular bed (Rosenfeld and Fixler, 1977; Reynolds and Redmer, 1995; Magness, 1998) ensuring optimum fetal growth and development (Reynolds and Redmer, 1995; Reynolds et al., 2010). During a normal pregnancy, uterine blood flow continues to rise throughout gestation. This is primarily achieved by a reduction in systemic vascular resistance and an increase in the cardiac output through an increase in heart rate and stroke volume (Rovinsky and Jaffin, 1966; Walters et al., 1966). Thus, for the systemic blood pressure to remain constant throughout pregnancy, the cardiac output increases and the total peripheral resistance decreases (Ford, 1982; Rosenfeld, 1984; Magness and Zheng, 1996; Magness, 1998). In fact, placental vascular insufficiency and elevated vascular resistance may lead to embryonic loss and impaired nutrient delivery to the fetus (Reynolds and Redmer, 1995, 2001; Reynolds et al., 2010).

During pregnancy, the placenta undergoes a variety of physiological changes to adapt to the various environmental stressors to drive fetal programming by regulating utero-placental blood flows to the fetus. This has been established by various models of placental insufficiency in sheep, pigs, and humans where uterine and umbilical blood flows were reduced (Meschia, 1983; Magness, 1998; Konje et al., 2003; Reynolds et al., 2006; Vonnahme et al., 2007; Wallace et al., 2008). There is increasing evidence that physiological alterations in the placental function and placental vasculature are controlled by several vasodilator and vasoconstrictor systems (Wang and Zhao, 2010). Additionally, the placental vascular tone could also be modulated by changes in receptor and gene expression of various angiogenic factors (Redmer et al., 2005; Reynolds et al., 2005 a, b, 2006; Luther et al., 2007; Vonnahme et al., 2007; Luther et al., 2008; Borowicz et al., 2009a).

Vasodilator pathways are imperative for maintaining optimum blood flow to the fetus. One fascinating example is the complex kallikrien kinin system (**KKS**) which is an integral part of the extensive vasodilatory network and is known for its interactions with other physiologic pathways such as the renin-angiotensin and complement pathways. The complex KKS produces a vasoactive kinin called bradykinin (**BK**) which lies at the center of this system. This portion of this review will specifically focus on BK production, its role in reproduction, and its mechanism of action for mediating vasorelaxation.

Kallikrein-kinin System

The kallikrein-kinin is a highly complex system where kallikrein represents a group of serine proteases which function to produce vasoactive peptides, the kinins, by acting on the endogenous substrates called kininogens (Müller-Esterl, 1989). Kallikrein has long been identified for its two different forms namely, the plasma and the tissue (Fiedler, 1979; Movat, 1979) kallikreins; which differ in several measures including the types of kinins liberated (Webster and Pierce, 1960; Webster, 1970; Fritz et al., 1977; Bhoola et al., 1979; Schachter, 1980; Fuller and Funder, 1986; Kalpan and Silverberg, 1987) which can result in either vasoconstriction or vasodilation. The released kinins are further classified into two types, based on the type of kininogen [high molecular weight kininogen (**HK**) and low molecular weight kininogen (**LK**)] (Jacobsen, 1966) stimulating its production. The HK stimulates the production of BK while the LK produces kallidin (**KD**) when acted upon by the plasma and the tissue kallikerins, respectively.

Plasma Kallikrein

Plasma kallikrein, or the Fletcher Factor, is recognized for its pivotal role in the intrinsic coagulation pathway and formation of BK. It is secreted in an inactive form called the plasma prekallikrein (Mandle et al., 1976; Reddigari and Kaplan, 1988; Bhoola et al., 1992); a single chain glycoprotein and a single gene product synthesized in the liver by hepatocytes (Asakai et al., 1987; Yu et al., 1998). Plasma prekallikrein cleaves the bound HK (Mandle et al., 1976; Reddigari and Kaplan, 1988; Bhoola et al., 1992) to liberate BK through a complex mechanism.

Factor XII, or Hageman factor, is the initiating protein and an essential part of the intrinsic coagulation pathway which gets activated upon contact with polyanionic surfaces or damaged blood vessels resulting in an autocatalytic cleavage of factor XII to form factor XIIa. Plasma prekallikrein and factor XI both complexed with HK (Mandle et al., 1976; Thompson et al., 1977; Wiggins et al., 1977; Sugo et al., 1980; Bhoola et al., 1992) are the two major substrates for factor XIIa. These complexes achieve a proper conformation by binding to the attachment sites on the HK domains resulting in cleavage of factor XI and prekallikrein to factor XIa and plasma kallikrein. While formation of factor XIa results in the continuation of the coagulation pathway (Ratnoff et al., 1961), plasma kallikrein exclusively cleaves HK to produce BK (Mori and Nagasawa, 1981; Kalpan and Silverberg, 1987). In addition to the above mentioned mechanism, BK is also produced by the binding of HK to endothelial cells. Following the binding, prekallikrein is readily cleaved to kallikrein resulting in the liberation of BK from HK (Nishikawa et al., 1992; Motta et al., 1998; Zhao et al., 2001).

Tissue Kallikrein (TK)

As the name suggests, tissue kallikreins are tissue specific enzymes encoded by a multigene family (Clements, 1989; Diamandis et al., 2000) with variable gene expressions in different tissue (Mason et al., 1983; Ashley and Mcdonald, 1985; Baker and Shine, 1985; Richards et al., 1989; Wines et al., 1989; Qin et al., 1991). The enzyme is expressed in several different tissues including lungs, kidney, intestine, brain, pancreas, spleen, adrenals, blood vessels, salivary and sweat glands (Bhoola et al., 1992; Mahabeer and Bhoola, 2000) with the number of genes forming the kallikrein family being highly variable amongst the different mammalian species (Mahabeer and Bhoola, 2000). However, only one gene encoding for the true form of tissue kallikrein (TK) involved in the release of vasopeptides has been identified. The enzyme was first isolated from porcine pancreatic kallikrein (Fiedler et al., 1976; Tschesche et al., 1979) and synthesized as prokallikrein (Kamada, 1990). The TK primarily functions to liberate a one residue longer kinin, Lys-BK or KD from its preferred substrate, the LK. Unlike the plasma kallikrein which exclusively cleaves HK to release BK, TK also has the capacity of producing BK by cleaving HK (Mahabeer and Bhoola, 2000).

Bradykinin: Its Receptors and Role during Gestation

The BK, a nonapeptide (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH), is a stimulator of vascular permeability (Bhoola et al., 1992) and a potent vasodilator which enhances blood flow to the uterus during estrous cycle (Resnik et al., 1976) and the utero-placenta during gestation (Albertini et al., 1980). Corthorn et al. (1997) have previously demonstrated an increase in TK expression in ovariectomized rats receiving subcutaneous doses of estrogen explaining the elevation observed in TK mRNA expression during proestrous stage of the estrous

cycle in rat (Corthorn and Valdés, 1994), and early pregnancy (Valdés et al., 1993). Since, one of the cleaved products of TK is BK and throughout gestation the developing placenta augments its production of estrogen becoming the major source of the steroid; it is essential to investigate the role of BK on placental vascular function during gestation which may contribute towards enhanced blood flow to the gravid uterus. In addition, BK also promotes angiogenesis, vascular permeability (Valdéz and Corthorn, 2011), and exerts its physiological effects on the cardiovascular system by acting on the vascular endothelium (Hong, 1980; Cocks et al., 1985; Palmer et al., 1987; Gryglewski et al., 1986; Mombouli et al., 1996; Bas et al., 2007).

The BK acts on the two cell surface G-protein coupled receptors, the BK1 receptor (**BK1R**) and the BK2 receptor (**BK2R**) (Bhoola et al., 1992; Leeb-Lundberg et al., 2005), the latter being the predominant receptor type (Bas et al., 2007). The kinin receptors share 36% sequence homology (Menke et al., 1994) and are members of the seven transmembrane family (Hess et al., 1992).

The locally produced kinin, BK is an autocoid which is readily cleaved by kininase I hence, making the accurate measurements of the peptide quite difficult. The action of kininase I on BK yields des-Arg-BK (Kurachi et al., 1977; Kaplan 2002) which selectively stimulates the BK1R, expressed as a result of chronic injury and inflammation (Regoli et al., 1980; Chai et al., 1996; Kaplan, 2002). On the contrary, the BK2R is expressed in various tissues and organs including the endothelial and vascular smooth muscle cells (Bhoola et al., 1992; Marceau et al., 1998; Christiansen et al., 2002; Marceau and Regoli, 2004; Souza et al., 2004; Moreau et al., 2005). Valdés et al. (2001) successfully localized the BK2R and TK in luminal and glandular epithelium, decidual cells, trophoblast invading arteries, endothelium and vascular, and myometrial smooth muscle in a human uterus. Since, these sites constitute the fetomaternal

interface, intervene in embryo attachment, implantation, placentation, and help in maintenance of placental blood flow and parturition; it establishes the investigation of KKS pathway particularly BK of monumental importance which highly likely aids these processes through vasodilation, increased vasopermeability, and stimulation of cell proliferation. The BK2R further promotes the release of endothelium derived vasodilatory mediators: nitric oxide (**NO**) (Furchgott and Zawadzki, 1980; Ignarro et al., 1981,1989; Moncada et al., 1991; Furchgott, 1995; Bian and Murad, 2003; Tanaka et al., 2004), prostacyclin (**PGI_2**) (Moncada et al., 1976; Coleman et al., 1994; Wise and Jones, 1996; Narumiya et al., 1999; Tanaka et al., 2004) and endothelium derived hyperpolarizing factors (**EDHF(s)**) (Nagao and Vanhoutte, 1993; McGuire et al., 2001; Busse et al., 2002; Suzuki, 2003; Triggle et al., 2003) for mediating vasorelaxation (Magness et al., 1993; Gainer et al., 1998). There is increasing evidence elucidating the important contributions of NO, PGI_2, and EDHF(s) in vasodilation since their role is up regulated as a result of an increase in endothelium stimulation during pregnancy (Poston et al., 1995; Gerber et al., 1998; Kenny et al., 2002; Bird et al., 2003).

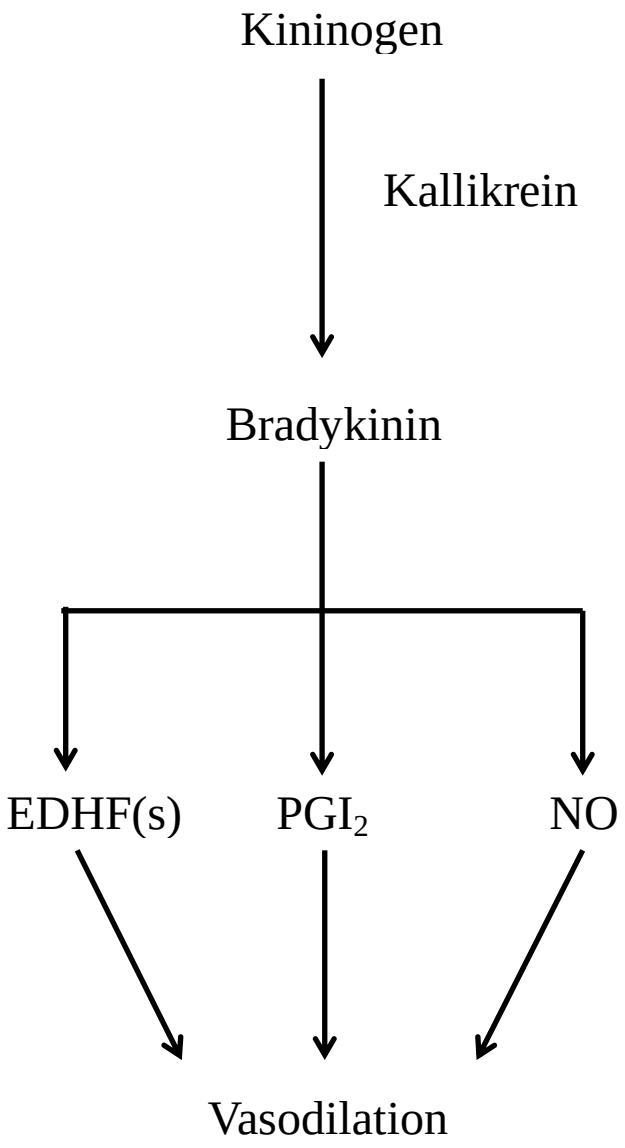

Figure 1.1. A simplified illustration of the kallikrein-kinin system (**KKS**). The KKS pathway involves the cleavage of kininogen by enzyme kallikrein for the production of a vasoactive kinin, bradykinin, a potent vasodilator. Bradykinin acts on kinin receptors on endothelial cells to liberate endothelial-derived hyperpolarizing factors (**EDHF**(**s**)), prostacyclin (**PGI2**), and nitric oxide (**NO**) for mediating vasorelaxation.

Nitric Oxide (NO)

The binding of BK to the BK2R triggers a cascade of events leading to vasodilation by the release of nitric oxide (NO) from the endothelial cells. Previously recognized as the endothelium derived relaxing factor (Cherry et al., 1982), NO is a free radical liberated as a

result of simulation of the Ca^{2+} dependent endothelial NO synthase (eNOS). The released NO diffuses from endothelial to smooth muscle cells activating soluble guanylate cyclase (sGC) causing an increase in the cGMP levels (Furchgott et al., 1981; Ignarro and Kadowitz, 1985; Ignarro, 1989). The elevated level of cGMP functions to activate protein kinase which in turn phosphorylates several proteins decreasing intracellular Ca^{2+} sensitivity; hence, stimulating dephosphorylation of myosin and producing vasorelaxation (Waldman and Murad, 1987; Carvajal et al., 2000). The vasodilatory effects of NO are blocked upon treatment with *N*(nitro)-L-arginine (NLA) (Pfeiffer et al., 1996).

Endothelium Derived Hyperpolarizing Factors (EDHF(s))

The endothelium derived hyperpolarizing factors or (EDHF(s)) are representative of a diverse group of chemical compounds comprising of L-citrulline, K^+ ion, H_2O_2, anandamide, and epoxyeicosatrienoic acids (Tanaka et al., 2004). These play a pivotal role in controlling resistance at the level of microvessels thereby influencing the systemic blood pressure (Suzuki et al., 1998; McGuire et al., 2001; Busse et al., 2002). Upon BK stimulation, one of the proposed mechanisms for mediating vasorelaxation, predominantly in the bovine coronary artery (Gauthier et al., 2005) involves the endothelium dependent hyperpolarization of vascular smooth cells through activation of the large conductance Ca^{2+} activated K^+ channels or the BK_{Ca} channels (Popp et al., 1996; Gauthier et al., 2005; Campbell and Falck, 2007; Campbell and Fleming, 2010). An increase in the intracellular Ca^{2+} level and membrane depolarization acts as a trigger for BK_{Ca} channels which when activated promote the efflux of K^+ ions from the cells causing membrane hyperpolarization (Nelson and Quayle, 1995; Jackson, 2000, 2005) and vasorelaxation. The vasodilatory effects of BK_{Ca} channels are attenuated upon treatment with iberiotoxin (IbTX) (Galvez et al., 1990).

Prostacyclins (PGI_2)

Prostacyclin (PGI_2) is an eicosanoid and a predominant metabolite of arachidonic acid released from the endothelial cells (Moncada et al., 1976, 1979) mediating vasodilation in vascular smooth muscle and inhibiting platelet aggregation (Robbins and Cotran, 1979). Due to its short half life various stable analogues of PGI_2 have been established to treat diseases such as pulmonary hypertension (Christman et al., 1992), hypoxia (Frisbee, 2001) and several cardiovascular diseases (Fink et al., 1999). Its active role in promoting relaxation has been established in human pulmonary artery and vein (Haye-Legrand et al., 1987; Walch et al., 1999), bovine coronary artery (Vegesna and Diamond, 1986), rabbit celiac, mesenteric and renal arteries (Ercan and Turkar, 1985), guinea pig aorta (Clapp et al., 1998; Ozaki et al., 1996) and dog cerebral, coronary, mesenteric, renal, and femoral arteries (Akiba et al., 1986). Once liberated from the endothelial cells by the action of the enzyme prostacyclin synthase, PGI_2 binds to IP receptor; belonging to the family of G-protein coupled receptors (Coleman et al., 1994; Narumiya et al., 1999; Wise and Jones 1996) and promotes paracrine signalling activating adenylyl cyclase and causing an increase in cAMP levels (Coleman et al., 1994; Narumiya et al., 1999; Wise and Jones 1996) leading to vasorelaxation. The elevated levels of cAMP also activates protein kinase A, leading to myosin relaxation and membrane hyperpolarization (Parkington et al., 1995; Corriu et al., 2001) ensuring vasodilation of vascular smooth muscle cells. The vasodilatory effects of PGI_2 are attenuated upon treatment with indomethacin (INDO) (Hart and Boardman, 1963).

Statement of the Problem

The concept of developmental programming explains that any insults or stressors to the maternal system *in utero* or during the neonatal period have the capacity of altering bodily systems and processes thus, producing life-long alterations in the postnatal life (Godfrey and Barker, 2000). Maternal suboptimal nutrition is one such stressor during pregnancy that is not only associated with a high risk of neonatal mortality and morbidity but can also program metabolic and reproductive changes in the offspring (Wallace, 1948; Wallace et al., 1999a; Godfrey and Barker, 2000). In fact, livestock in the United States are often under a poor nutritional environment during pregnancy (Wu et al., 2006). There is increasing evidence that maternal under-nutrition during gestation in humans and livestock causes IUGR which can lead to stunted placental-fetal growth and development (Barker and Clark, 1997; Bell and Ehrhardt, 2002). While, it is evident that IUGR poses a significant threat to the fetal growth and development, designing potential therapeutic strategies to overcome IUGR remains problematic.

The placenta aids in nutrient transfer which is primarily achieved by regulating the utero-placental blood flows (Reynolds and Redmer, 1995) to the fetus. Therefore, an abnormal placentation caused as the result of IUGR leads to a decrease in utero-placental blood flows (Bell, 1999; Hay Jr. et al., 1999) resulting in fetal (Bell, 1999; Hay Jr. et al., 1999) and trophoblast (Myatt, 2006) hypoxaemia, fetal hypoglycaemia, and pre-eclampsia (Myatt, 2006). Since in the bovine, maximal placental growth occurs during the first half of gestation, followed by the fetal growth during the last half of gestation (Ferrell et al., 1976; Ferrell and Ford, 1980; Reynolds et al., 1990; Reynolds and Redmer, 1995), this makes the fetal growth trajectory highly sensitive to the timing, duration, and severity of the maternal nutritional insult (Robinson et al., 1999).

There are several studies examining the role of maternal diet on fetal (Charlton and Johengen, 1985; Holst et al., 1986, 1992; Faichney and White, 1987; Bloomfield et al., 2000; Vonnahme et al., 2003; Scheaffer et al., 2004; Redmer, 2004; Lemley et al., 2012) and placental (Charlton and Johengen, 1985; Faichney and White, 1987; McCrabb et al., 1991, 1992a, 1992b; Heasman et al, 1998; Redmer, 2004; McMullen et al., 2005) growth in the ewes; however, due to the evident differences in the placental growth and vascularity in the two species, it is imperative that the ovine and the bovine species are studied separately and compared with caution.

While, there are a few studies elucidating the role of maternal suboptimum nutrition during pregnancy in bovine; studies investigating the effects of maternal under-nutrition followed by realimentation on the bovine placental vasculature are limited (Perry et at., 1999; Vonnahme et al., 2007; Zhu et al., 2007). Furthermore, the precise mechanisms mediating the effects of maternal nutrient restriction and/or realimentation on the bovine placental function and particularly on the caruncular and cotyledonary arterial vasculature have not been revealed. Therefore, the purpose of our research is to provide a better understanding of the bovine placental vascular remodeling during gestation which regulates the fetal growth and development.

We hypothesize that global maternal nutrient restriction during early and mid-gestation and restriction followed by realimentation in pregnant beef cows would alter placental arterial vascular function due to changes in placental arterial sensitivity to bradykinin. Further, we also hypothesize that placental arterial vasodilation is mediated at least partly through the bradykinin 2 receptor and that global maternal nutrient restriction and realimentation may impact the pathways mediating bradykinin induced vasodilation in the placental arteries.

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CHAPTER 2. INFLUENCE OF MATERNAL NUTRIENT RESTRICTION DURING EARLY PREGNANCY ON PLACENTAL VASCULAR FUNCTION IN PREGNANT BEEF COWS

Abstract

We hypothesize that maternal nutrient restriction during early gestation would alter the caruncular (**CAR**) and cotyledonary (**COT**) placental artery sensitivity to bradykinin (**BK**) induced relaxation. Further, we hypothesized the placental arterial vasodilation is mediated at least partly through the bradykinin 2 receptor (**BK2R**) and its pathways which may be impacted by the maternal diet. To examine the effects of maternal nutrient restriction on CAR and COT artery vasoactivity during early gestation, multiparous beef cows were randomly assigned to either 100% (**C**; n = 6) or 60% NRC requirements (**R**; n = 4) on d 30 of gestation. At d 85 of gestation cows were euthanized and arteries that terminated into the maternal (CAR artery) and fetal portions (COT artery) of the placentome were dissected to be used for in vitro vasoreactivity assays and mRNA expression. The endothelium-intact CAR arterial rings were loaded in an organ bath chamber and contracted with NE (10^{-6} M) and the concentration response curve (**CRC**) to BK was obtained. In addition, a BK CRC was obtained for rings which were incubated for 30 min with inhibitors to: 1) BK2R, icatibant acetate (**HOE 140**; 10^{-6} M); 2) large conductance Ca^{2+} activated K^{+} channels, iberiotoxin (**IbTX**; 10^{-7} M); 3) endogenous nitric oxide synthase, *N*(nitro)-L-arginine, (**NLA**; 10^{-5} M); and 4) prostacyclins (PGI_2), indomethacin (**INDO**; 10^{-5} M). Additionally, endothelium-intact COT arterial rings were contracted with U46619 (10^{-6} M) and the effect of BK and the above mentioned inhibitors were studied with the exception of PGI_2. The CAR arterial rings with no inhibitor (**NI**) were influenced by the maternal diet and arteries from the R cows were more sensitive ($P < 0.0001$) to BK induced relaxation

compared with the C cows. An overall effect of maternal nutrition was observed which blocked ($P < 0.0001$**)** BK mediated vasodilation in the C and R arteries incubated with HOE 140. In the CAR placental arteries, the BK dose response curves had a significant treatment by dose interaction in the presence of IbTX ($P \leq 0.04$) and NLA ($P \leq 0.04$) which blocked relaxation in the C and the R arteries. An effect of maternal diet was observed in the presence of INDO which inhibited relaxation ($P < 0.0001$) in the C and the R arteries. Our findings indicate that the downstream pathways activated by BK are important for vasodilation in the CAR placental arteries. The COT arteries with NI had a significant treatment by dose interaction and arteries from R cows were more sensitive ($P = 0.04$) to the BK induced relaxation compared with C cows. A significant treatment by dose interaction ($P < 0.0001$) was observed in C cows in the presence of HOE 140 while an effect of diet ($P = 0.004$) was observed in the R cows which blocked relaxation in the COT arteries. The C arteries were more sensitive ($P = 0.003$) to BK induced relaxation in the presence of IbTX while no effect was observed in the R arteries. In the COT placental artery, a significant treatment by dose interaction was observed in the presence of NLA and relaxation was completely abolished ($P < 0.0001$) across treatments. The mRNA expression of BK2R was not altered ($P \geq 0.51$) by the maternal diet during early gestation. Results from this study indicate that maternal nutrient restriction does not negatively impact fetal or placental growth and it appears to alter placental vascular function, which could alter nutrient availability to the conceptus.

Key words: bradykinin, cow, placenta, vascular function

Introduction

Maternal nutrient requirements might appear trivial during early gestation but it plays a significant role in the placental-fetal development since the earliest stages of embryonic development (Bishonga et al., 1996; Sugden, 2002; Waterland, 2004). Studies in ewes and rodents suggest that poor maternal nutrition at the time of conception leads to reduced number of cells in the inner cell mass (Robinson et al., 1994; Walker et al., 1996) which often leads to postnatal complications (Kwong et al., 2000; Barker, 2001). Nutritionally challenged women (Barker et al., 1993; Godfrey et al., 1996; Ravelli et al., 1988; Godfrey and Barker, 2000) and ewes (Whorwood et al., 2001) during the first half of gestation have been found to produce longer and thinner babies. Similarly, early nutrient restriction in the bovine has been reported to cause a decrease in fetal and placentome weight (Vonnahme et al., 2007) and an increase in the cotyledonary arteriolar vascularity (Zhu et al., 2007) at the end of nutrient restriction. These studies strongly indicate that alterations in placental function might be a result of maternal under-nutrition during early gestation.

Bradykinin (**BK**) is potent vasodilator associated with enhanced blood flow to the utero-placenta during gestation (Albertini et al., 1980). The exact mechanisms of BK mediated vasodilation in the bovine placental arteries are not known. Further, the impact of maternal nutrient restriction during early gestation on these mechanisms has not been investigated. Therefore, we hypothesize that global maternal nutrient restriction during early gestation in pregnant beef cows would alter placental arterial vascular function due to changes in placental arterial sensitivity to BK. Further, we hypothesize that placental arterial vasodilation is mediated at least partly through the BK2R and that global maternal nutrient restriction may impact the pathways mediating BK induced vasodilation in the placental arteries. The objective of this study

was to examine the effect of maternal nutrient restriction on mechanisms of BK induced vasorelaxation in the bovine placental arteries.

Materials and Methods

All procedures involving animals were approved by the North Dakota State University (NDSU) Animal Care and Use Committee (#A10001).

Animals, Diets, and Breeding

A total of 46 non-lactating, multiparous crossbred beef cows of similar genetic background were synchronized using a Select Synch plus progesterone insert (CIDR; Pfizer Animal Health, New York, NY) and fixed-time AI (TAI) protocol. At the NDSU Beef Research and Teaching Unit (Fargo, ND), cows were assigned to 1 of 6 breeding groups with breeding dates ranging from July 13th to October 24th 2011. Cows received GnRH (100 µg as 2 mL of Factrel i.m.; Fort Dodge Animal Health, Fort Dodge, IA) and a CIDR on d 0. On d 7 CIDR devices were removed, and cows were given an injection of PGF2α (25 mg as 5 mL of Lutalyse i.m., Pharmacia & Upjohn Co, Kalamazoo, MI). Estrotect Heat Detectors (Rockway Inc., Spring Valley, WI) were used to monitor estrous behavior for a minimum of 72 h. Artificial insemination was performed utilizing the AM/PM rule 12 h after the first detected estrus. Cows not detected in estrus after 72 h received a second GnRH injection and TAI was performed. Inseminated cows were transported to the Animal Nutrition and Physiology Center (ANPC; Fargo, ND) within 3 d post-insemination. From arrival at ANPC until confirmed pregnant, cows were grouped in pens (n = 4 to 5/pen) and trained to use the Calan gate feeding system. At this time, all cows were fed chopped grass hay [8.02% crude protein, 69.2% neutral detergent fiber, 41.5% acid detergent fiber, and 57.9% total digestible nutrients (dry matter basis)] and a mineral and vitamin supplement to meet net energy recommendations for maintenance and fetal growth and to meet

or exceed recommendations for metabolizable protein (MP), minerals, and vitamins (NRC, 2000) until pregnancy was confirmed. Hay net energy of maintenance concentration was predicted using equations described by Weiss (1993) and NRC (2000).

On d 27 and 28 post-insemination, pregnancy was confirmed via transrectal ultrasonography (500-SSV; Aloka, Tokyo, Japan) using a linear transducer probe (5 MHz). Non-pregnant cows restarted the same breeding protocol; cows were only subjected to AI twice during the experiment. On d 30 of pregnancy, cows (initial BW = 620.5 ± 11.3 kg, BCS = 5.1 ± 0.1) were randomly assigned to either 100% (**C**; n = 6) or 60% NRC requirements (**R**; n = 6). On d 85 of gestation cows were euthanized and arteries terminating into the maternal (caruncular, **CAR**) and the fetal (cotyledonary, **COT**) portions of the placentome were dissected to be used for in vitro vasoreactivity assays and mRNA expression.

The control diet consisted of grass hay fed to meet 100% NE recommendations for maintenance and fetal growth (NRC, 2000) and to meet or exceed MP, mineral, and vitamin recommendations. Nutrient restricted cows received 60% of the same control hay diet. Cows were individually fed once daily in a Calan gate system at 1000 and had free access to water. The mineral and vitamin supplement (Trouw dairy VTM with optimins; Trouw Nutrition International, Highland, IL) was top-dressed 3 times per week at a rate of 0.18% of hay dry matter intake to meet or exceed mineral and vitamin requirements relative to dietary net energy intake (NRC 2000). Cows were weighed weekly at approximately 0800 throughout the experiment and dietary intake was adjusted relative to BW.

Tissue Collection and Preparation

Experimental drugs and solutions used in organ chamber studies

The following drugs were used: Bradykinin (**BK**; Sigma Chemical Co., St Louis, MO, USA), norepinephrine (**NE**; Sigma); 9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (**U46619**; Cayman Chemical Company, Ann Arbor, MI, USA), icatibant acetate (**HOE 140**; Sigma), iberiotoxin (**IbTX**; Tocris Biosciences, Minneapolis, MN, USA), *N*(nitro)-L-arginine (**NLA**; Sigma) and indomethacin (**INDO**; Sigma). Drug solutions were prepared daily, kept on ice and protected from light until use. The BK was initially dissolved in distilled water followed by subsequent dilution preparations in distilled water and INDO was dissolved in 100mM Na_2CO_3 and the mixture was sonicated for 30 minutes. All the other drugs preparations were dissolved in distilled water. Drugs were added to organ chambers in volumes not greater than 0.2 ml/ 25 ml or 5 ml of physiological saline solution (PSS). Drug concentrations are reported as final molar concentration in the organ chamber. The PSS is a modified Krebs solution with the following composition (in mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and glucose, 11.1 (pH = 7.4).

Organ chamber studies

The CAR arterial rings were suspended in water-jacketed organ chambers filled with 25 ml of PSS. The organ chamber solution was aerated with a mixture of 95% O_2/5% CO_2 and maintained at a temperature of at 37˚C throughout the experiment. Five CAR arterial rings were suspended by means of two fine stainless-steel wire clips that passed through the lumen; one clip was anchored inside the organ chamber, the other was connected to a force transducer (Model FT03, Grass Instrument Company, Quincy, MA, USA). The arterial ring preparations were

allowed to equilibrate for 45 min. Isometric tension in the vessels was measured and recorded on a Grass polygraph. The rings were stretched progressively at an interval of 10 min each to obtain an optimal point of their length–active tension relationship, as determined by the contractile response generated in response to KCl (20 mM). Every application was followed by rinsing the arterial preparation twice with PSS. After this procedure, the preparations were allowed to equilibrate at their optimal length for at least 30 min prior to further exposure to any vasoactive substances. The presence of an intact endothelium was confirmed in each preparation by treating arterial rings to the endothelium-dependent vasodilator, BK (10^{-7} M). A successful vasodilation established the presence of a functional endothelium and the arterial rings which were suspected of a damaged endothelium were replaced with a new arterial segment. The preparations were then rinsed three times for 45 min with PSS until they relaxed to baseline tension.

Relaxation of placental arteries was studied in rings contracted with NE (10^{-6} M). After the NE induced contraction had reached a stable plateau, relaxation responses to increasing concentrations of BK (10^{-10} – 10^{-6} M) were obtained. In addition, BK concentration response curves (**CRCs**) were obtained for rings which were incubated for 30 min with a BK2 receptor (**BK2R**) antagonist, HOE 140 (10^{-6} M); large conductance Ca^{2+} activated K^+ channels (**BK_{Ca} channels**), IbTX (10^{-7} M); endothelial nitric oxide synthase (**eNOS**) inhibitor, NLA (10^{-5} M); and prostacyclin (**PGI_2**) inhibitor, INDO (10^{-5} M).

In a separate series of experiments, four COT placental arterial rings were suspended in organ chambers of a multi wire myograph system (DMT- 620M, Ann Arbor, MI, USA) filled with 5 ml of PSS aerated with mixture of 95% O_2/5% CO_2. A heat exchanger maintained the temperature of the circulating PSS constant at 37˚C. Preparations were threaded onto two wires passing through the lumen of the artery. The wires were secured to a tension transducer which

measured isometric tension in the vessel and a displacement device which allowed for the control of ring circumference.

The arterial ring preparations were allowed to equilibrate for 30 min. Isometric tension was measured and directly recorded on a Power Lab data acquisition system (AD instruments, Colorado Springs, CO). The rings were stretched progressively at an interval of 10 min each to obtain an optimal point of their length–active tension relationship, as determined by the contractile response generated in response to KCl (20 mM). Every application was followed by rinsing the arterial preparation twice. The organ chamber solution was drained each time and replaced with warm PSS. After this procedure, the preparations were allowed to equilibrate at their optimal length for at least 30 min prior to further exposure to any vasoactive substances. The presence of an intact endothelium was confirmed in each preparation by treating arterial rings to the endothelium-dependent vasodilator, BK (10^{-7} M). Successful vasodilation established the presence of a functional endothelium and arterial rings suspected of a damaged endothelium were replaced with a new arterial segment. The preparations were then rinsed three times for 40 min with PSS until they relaxed to baseline tension.

Relaxation of placental arteries was studied in rings contracted with U46619 (10^{-6} M), a thromboxane A2-mimetic. After the U46619 induced contraction reached a stable plateau, relaxation responses to increasing concentrations of BK (10^{-10} – 10^{-6} M) were obtained. In addition, BK CRCs were obtained for rings which were incubated for 30 min with a BK2R antagonist, HOE 140 (10^{-6} M); BK_{Ca} channels, IbTX (10^{-7} M); and eNOS inhibitor, NLA (10^{-5} M). The effects of PGI_2 in the COT placental arteries were not examined due to space.

Receptor Quantification

Tissue extraction

On d 85 of gestation, arteries terminating in the CAR and COT portions of the placentome were isolated for BK2R mRNA expression analysis. The placental vessels were dissected free from the surrounding placental tissue. The dissected CAR and COT placental arteries were frozen in liquid nitrogen and stored at -80˚C for subsequent mRNA analysis.

Quantification of mRNA

Frozen arterial tissues (30mg) were suspended in commercially available lysis buffer (Qiagen, Valencia, CA, USA) and directly homogenized using a Polytron homogenizer (Kinematica Type PT10/35, Brinkman Instrument Inc., Westbury, NY, USA) fitted with a 7mm generator. Total mRNA was extracted using a QIAshredder spin column and an RNeasy Mini Kit (Qiagen) following manufacturer's suggested protocol. RNA was quantified by measuring absorbance on a Nanodrop 2000c spectrophotometer.

For each CAR and COT placental arterial tissue, mRNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Genomic DNA was removed and Quantitative RT-PCR on an ABI 7000 sequence detection system (Applied Biosystem, Foster City, CA, USA) was performed. The probe and primer sequences for BK2R (ABI, pre-developed assay reagent- primers and VIC- TAMRA probe) are as follows:

Probe: 5'–6FAMCAACAGCTGCCTCAACCCCCTGGTAMRA– 3'

(FAM – Reporter, TAMRA – Quencher)

BK2R– FP: 5'–TCGCGTCCTTTGTGGCTTAC– 3'

BK2R– RP: 5'–CGCTTGCCCACGATCAC– 3'

All reactions were carried out in a 12.5 µL reaction mixture containing, 2µl of cDNA and 10.5 µL of PCR Master Mix (TaqMan universal PCR master mix (2X), forward primer (1µM), reverse primer (1µM), probe (200nM), and ddH_20). Dissociation curves confirmed a single amplification species for BK2R. The endogenous control, 18s rRNA was used for normalization of the raw data.

Data Analysis

Vasoreactivity assays and quantification of mRNA

Data were analyzed within a day. Relaxation responses are expressed as a percentage of the initial tension induced by NE in CAR and U46619 in COT placental arteries. 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. The EC_{50} values were converted to the negative logarithms and expressed as -log molar EC_{50} (**p*D*$_2$**). The effects of maternal diet on dependent variables were tested using PROC GLM of SAS (SAS software version 9.2, SAS Institute, Cary, NC) and means were separated using the LSMEANS statement. The class statement included the effects of dietary treatment, dose, and the dietary treatment by dose interaction within a tissue (COT or CAR) for responses to BK in the presence and absence of the various inhibitors tested. The effect of dietary treatment was also tested on E_{max} and pD_2 data. Gene expression was quantified as a ratio of BK2R to 18s rRNA. The effects of maternal diet on dependent variables were tested using PROC GLM of SAS and means were separated using the LSMEANS statement. The model statement included: BK2R to 18s rRNA ratio, delta CT, and the percentage change from control. Results are expressed as LSmeans ± SEM and *n* refers to the number of animals from which the placental arteries were derived. Data from some

animals were not used in the study if an artery was unresponsive or was found to be an overall outlier.

Results

Caruncular Arterial Responses

The treatment by dose interaction on the BK response curve was not significant (P = 0.95) in the CAR arteries (Figure 2.1A) but, there was an overall effect of maternal nutrition during early in gestation. A similar pattern of relaxation was observed in arteries from the C-NI and the R-NI cows where R-NI cows were more sensitive ($P < 0.0001$) to BK ($10^{-10} – 10^{-6}$ M) induced vasodilation than the C-NI cows. While there was a tendency for a lower (P = 0.07) E_{max} value in the arteries from the R-NI cows (18.38 ± 11.97) vs the C-NI cows (52.54 ± 10.75), the pD_2 values were not influenced (P = 0.74; 7.95 ± 0.41 and 7.74 ± 0.46 for C-NI and R-NI, respectively) by the maternal diet for BK CRC.

There was no treatment by dose interaction when arteries were incubated with HOE 140; however, an overall effect of maternal nutrition was observed which blocked BK mediated vasodilation in the C-HOE 140 vs C-NI ($P < 0.0001$) arteries (Figure 2.1B) and R-HOE 140 vs R-NI ($P < 0.0001$) arteries (Figure 2.1F). The E_{max} values tended to be higher in the presence of HOE 140 in the C-HOE 140 (P = 0.06, 84.91 ± 10.39) vs C-NI (52.54 ± 10.39) arteries and R-HOE 140 (P = 0.08, 65.27 ± 15.89) vs R-NI (18.38 ± 15.89) arteries; while the pD_2 values did not significantly differ among the C (P = 0.68; 7.95 ± 0.50 vs 7.63 ± 0.56 for C-NI and C-HOE 140, respectively) and the R (P = 0.73; 7.74 ± 0.69 vs 8.12 ± 0.79 for R-NI and R-HOE 140, respectively) treatment groups for HOE 140.

In the CAR placental arteries, the BK CRCs had a significant treatment by dose interaction in the presence of IbTX. Relaxation was abolished in the C-IbTX vs C-NI ($P = 0.001$) (Figure 2.1C) and R-IbTX vs R-NI ($P = 0.04$) arteries (Figure 2.1G). Incubation with IbTX led to an increase in the E_{max} values in the C-IbTX ($P = 0.004$; 93.91 ± 7.4) vs C-NI (52.54 ± 8.11) arteries and the R-IbTX ($P = 0.006$; 104.92 ± 14.84) vs R-NI (18.38 ± 14.84) arteries; while the pD_2 values did not differ among the C ($P = 0.36$; 7.95 ± 0.37 vs 7.41 ± 0.42 for C-NI vs C-IbTX, respectively) and R ($P = 0.68$; 7.74 ± 0.53 vs 8.07 ± 0.53 for R-NI vs R-IbTX, respectively) treatment groups for IbTX.

When incubated with NLA, a treatment by dose interaction was observed and C-NLA ($P = 0.02$) and R-NLA ($P = 0.04$) arteries did not relax when compared with C-NI (Figure 2.1D) and R-NI (Figure 2.1H) arteries. Moreover, the maternal diet had a significant impact on the E_{max} values in the presence of NLA resulting in higher E_{max} values in the C-NLA ($P = 0.024$; 85.56 ± 8.2) vs C-NI (52.54 ± 8.98) and R-NLA ($P = 0.005$; 76.86 ± 9.53) vs R-NI (18.38 ± 9.53) arteries. The BK CRCs pD_2 values for C ($P = 0.37$; 7.95 ± 0.27 vs 8.32 ± 0.27 for C-NI vs C-NLA, respectively) and R ($P = 0.37$; 7.74 ± 0.50 vs 8.43 ± 0.50 for R-NI vs R-NLA, respectively) arteries did not differ for NLA.

For CAR placental arteries incubated with INDO, the treatment by dose interaction was not significant ($P \geq 0.23$); however, an effect of maternal diet was observed in the presence of INDO which inhibited relaxation in the C ($P < 0.0001$) (Figure 2.1E) and R ($P < 0.0001$) cows (Figure 2.1I). Maternal diet also had an impact on the E_{max} values which were higher in C-INDO ($P = 0.05$; 83.25 ± 9.0) and R-INDO ($P = 0.01$; 67.13 ± 9.75) arteries vs C-NI (52.54 ± 9.87) and R-NI (18.38 ± 10.89) arteries. The BK CRCs pD_2 values did not significantly differ among the C

(P = 0.37; 7.95 ± 0.34 vs 8.41 ± 0.34 for C-NI and C-INDO, respectively) and R (P = 0.93; 7.74 ± 0.56 vs 7.82 ± 0.56 for R-NI and R-INDO, respectively) treatment groups for INDO.

Cotyledonary Arterial Responses

In the COT placental arteries, there was a treatment by dose interaction and an effect of treatment was observed when arteries were treated with BK (Figure 2.2A). The C-NI and R-NI arteries responded similarly until the 3×10^{-7} M dose of BK after which the R-NI arterial rings showed higher sensitivity (P = 0.04) to BK (10^{-10} – 10^{-6} M) induced vasodilation compared with the C-NI arterial rings. While the dietary treatment led to a lower (P < 0.01) E_{max} value in the arteries from the R-NI (42.77 ± 9.04) vs the C-NI (83.08 ± 7.38) cows the pD_2 values did not differ (P = 0.07; 8.13 ± 0.69 vs 5.90 ± 0.69 for C-NI and R-NI, respectively) for BK CRC.

The COT arterial rings incubated with HOE 140 had a significant treatment by dose interaction; however, this effect was only observed in C (P < 0.0001) cows. The C-NI and C-HOE 140 arteries responded similarly to BK until the 3×10^{-8} M dose of BK (Figure 2.2B). Beginning at the 10^{-7} M dose of BK, vasodilation continued to be completely abolished (i.e. 100%) in the C-HOE arteries whereas, the C-NI arteries relaxed. Furthermore, the maternal diet led to a higher E_{max} value in the presence of HOE 140 (P < 0.0001; 83.08 ± 7.35 vs 148.92 ± 7.35 for C-NI and C-HOE 140, respectively) in the C arteries, while the pD_2 values (8.13 ± 0.71 vs 8.18 ± 0.64 for C-NI and C-HOE 140, respectively) were similar.

In the R arteries, there was a tendency for a treatment by dose interaction (P = 0.07) and an effect of the dietary treatment (P = 0.004) was observed upon incubation with HOE 140 (Figure 1.2E). The R-NI and R-HOE 140 arteries responded similarly to BK until the 3×10^{-7} M dose of BK (Figure 2.2E). Beginning at the 10^{-6} M dose of BK, relaxation was inhibited in the C-

HOE 140 arteries whereas, the C-NI arteries showed higher sensitivity to BK induced relaxations. The BK dose response E_{max} values tended to be higher in the presence of HOE 140 (P = 0.07; 42.77 ± 16.75 and 94.25 ± 16.75 for R-NI and R-HOE 140, respectively) in the R placental arteries while, the pD_2 values (P = 0.17; 5.90 ± 0.62 vs 7.27 ± 0.62 for R-NI and R-HOE 140, respectively) were not influenced by the maternal diet for HOE 140.

The C arterial rings, had a significant treatment by dose interaction (P = 0.003) in the presence of IbTX (Figure 2.2C). Similar patterns of relaxations were observed until the 10^{-8} M dose of BK, after which the BK induced relaxation, was enhanced in the C-IbTX arteries vs the C-NI arteries. The C-NI arteries (83.08 ± 9.55) had a greater (P = 0.03) E_{max} value than the C-IbTX (47.69 ± 9.55) arteries while the pD_2 values did not differ (P = 0.11; 8.13 ± 0.61 vs 6.73 ± 0.49 for C-NI and C-IbTX, respectively). In the R arterial rings, there was no treatment by dose interaction (P = 0.98) but, a tendency for a treatment effect (P = 0.09) and an effect of dose was significant (P < 0.0001) where the R-NI and R-IbTX arteries responded similarly to BK induced vasodilation (Figure 2.2F). Dietary treatment had no impact on the E_{max} values (P = 0.94; 42.77 ± 7.92 vs 43.62 ± 7.92 for R-NI and R-IbTX, respectively) and pD_2 values (P = 0.34; 5.90 ± 0.32 vs 6.37 ± 0.32 for R-NI and R-IbTX, respectively) in the R arteries in the presence of IbTX.

In the COT placental artery, a significant treatment by dose interaction was observed when arterial rings were incubated with NLA. Regardless of maternal nutrition, relaxation was completely abolished (i.e. 100%) in the C (P < 0.0001) (Figure 2.2D) and R (P < 0.0001) (Figure 2.2G) arteries. Moreover, the E_{max} values were significantly higher (P < 0.0001; 83.08 ± 9.22 vs 164.33 ± 9.22 for C-NI and C-NLA, respectively; P < 0.0001; 42.77 ± 8.00 vs 148.69 ± 8.00 for R-NI and R-NLA, respectively) in COT arteries treated with NLA for both the C and the R treatment groups. Additionaly, dietary treatment had an effect on the pD_2 values in the R cows

where a leftward shift (P = 0.0004; 5.90 ± 0.23 vs 8.26 ± 0.23 for R-NI and R-NLA, respectively) was observed in BK CRC in the presence of NLA while, the pD_2 values in the C cows (P = 0.95; 8.13 ± 0.64 vs 8.19 ± 0.52 for C-NI and C-NLA, respectively) were similar.

Caruncular and Cotyledonary Arterial Responses to Bradykinin

The CRCs comparing the effects of BK on the C-CAR vs C-COT and R-CAR vs R-COT are depicted in figures 2.3A and 2.3B. A treatment by dose interaction was observed and the sensitivity to BK induced relaxation in the CAR placental arteries was enhanced in C-CAR (P = 0.02) and R-CAR (P = 0.007) vs C-COT and R-COT arteries. There was an effect of dietary treatment on the E_{max} values ($P < 0.05$) in the C-CAR (52.54 ± 9.94) vs C-COT (83.08 ± 9.07) arteries while the pD_2 values (P = 0.85; 7.95 ± 0.61 vs 8.13 ± 0.69 for C-CAR and C-COT, respectively) did not differ. Maternal nutrition also had a significant effect on the pD_2 values (P = 0.02) in the R-CAR (7.74 ± 0.43) vs R-COT (5.90 ± 0.43) arteries while the E_{max} values were similar (P = 0.12; 18.38 ± 9.54 vs 42.77 ± 9.54 for R-CAR and R-COT, respectively).

BK2R mRNA Expression

Maternal dietary treatment did not influence the mRNA expression of BK2R in the CAR (P = 0.51; 1.18, 1.29 ± 0.11 arbitrary units for C and R, respectively) and the COT (P = 0.97; 1.25, 1.26 ± 0.14 arbitrary units for C and R, respectively) placental arteries.

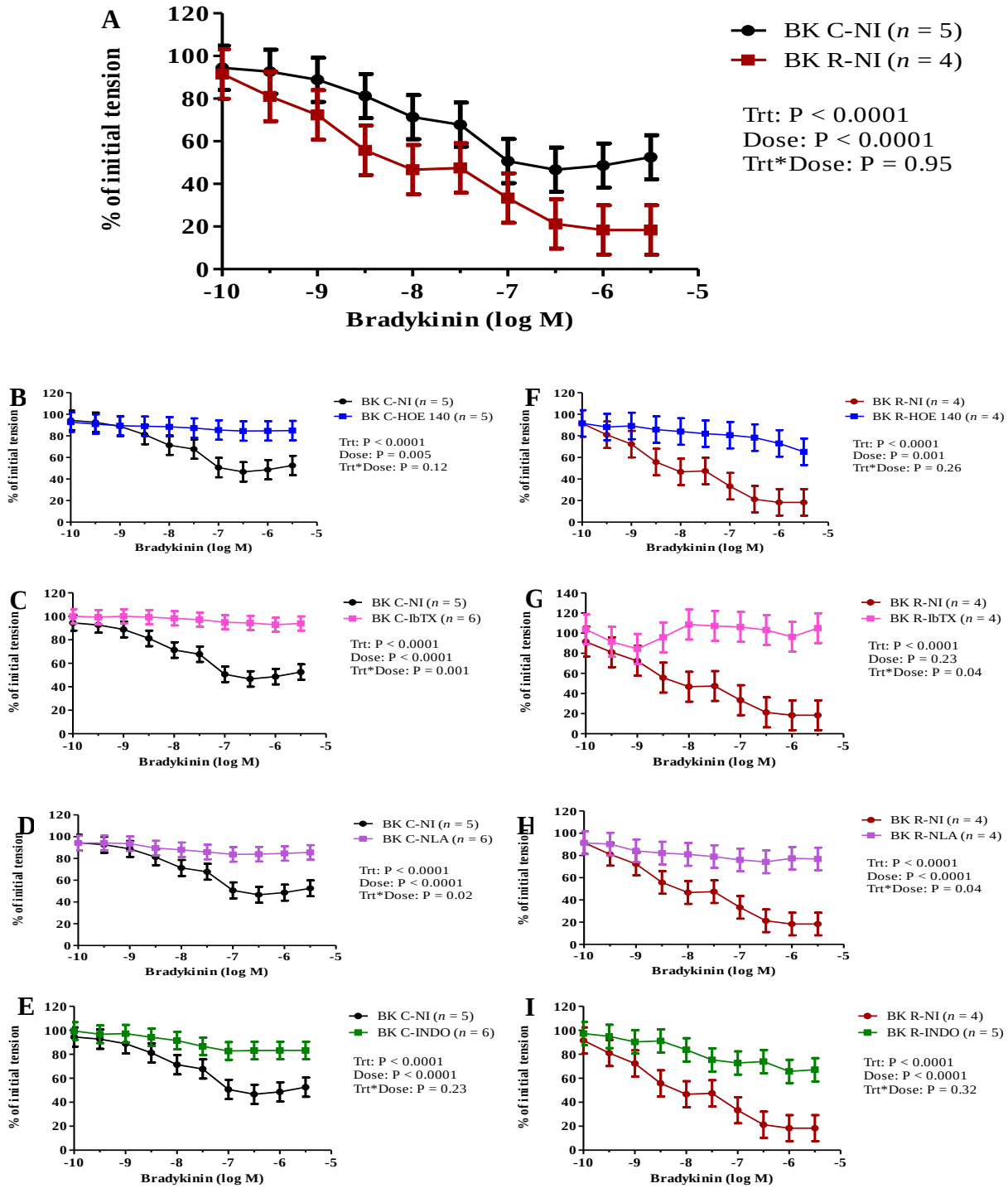

Figure 2.1. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in carnuncular (**CAR**) arteries (**A**) in the absence of an inhibitor (no inhibitor, **NI**), (**B, F**) after incubation with icatibant acetate (**HOE 140**), (**C, G**) after incubation with iberiotoxin (**IbTX**), (**D, H**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**E, I**) after incubation with indomethacin (**INDO**). Animals received either 100% (**C**) or 60% (**R**) of NRC requirements. Diets were fed from d 30 to d 85 of gestation.

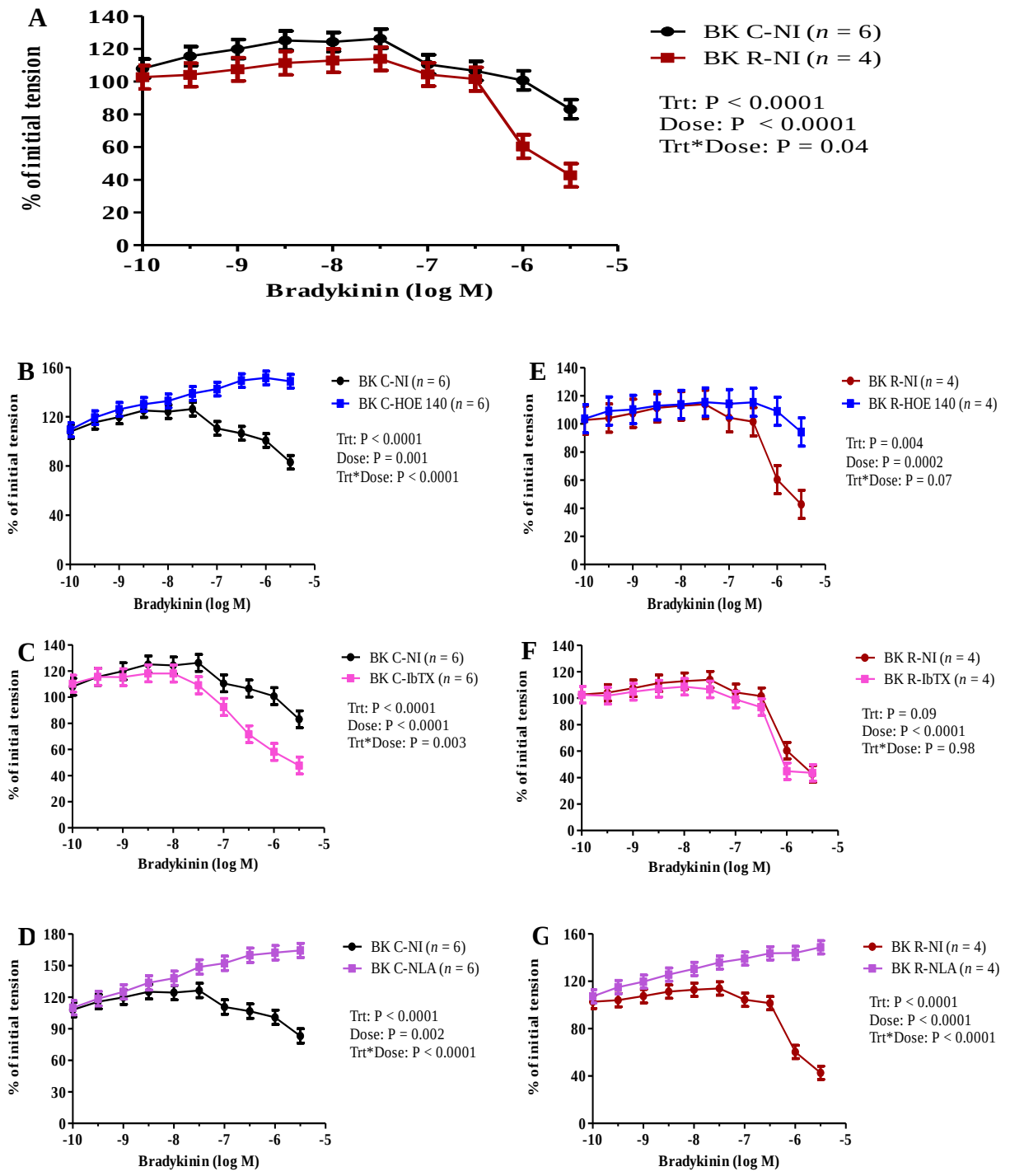

Figure 2.2. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries (**A**) in the absence of an inhibitor (no inhibitor, **NI**), (**B, E**) after incubation with icatibant acetate (**HOE 140**), (**C, F**) after incubation with iberiotoxin (**IbTX**), and (**D, G**) after incubation with *N*(nitro)-L-arginine (**NLA**). Animals received either 100% (**C**) or 60% (**R**) of NRC requirements. Diets were fed from d 30 to d 85 of gestation.

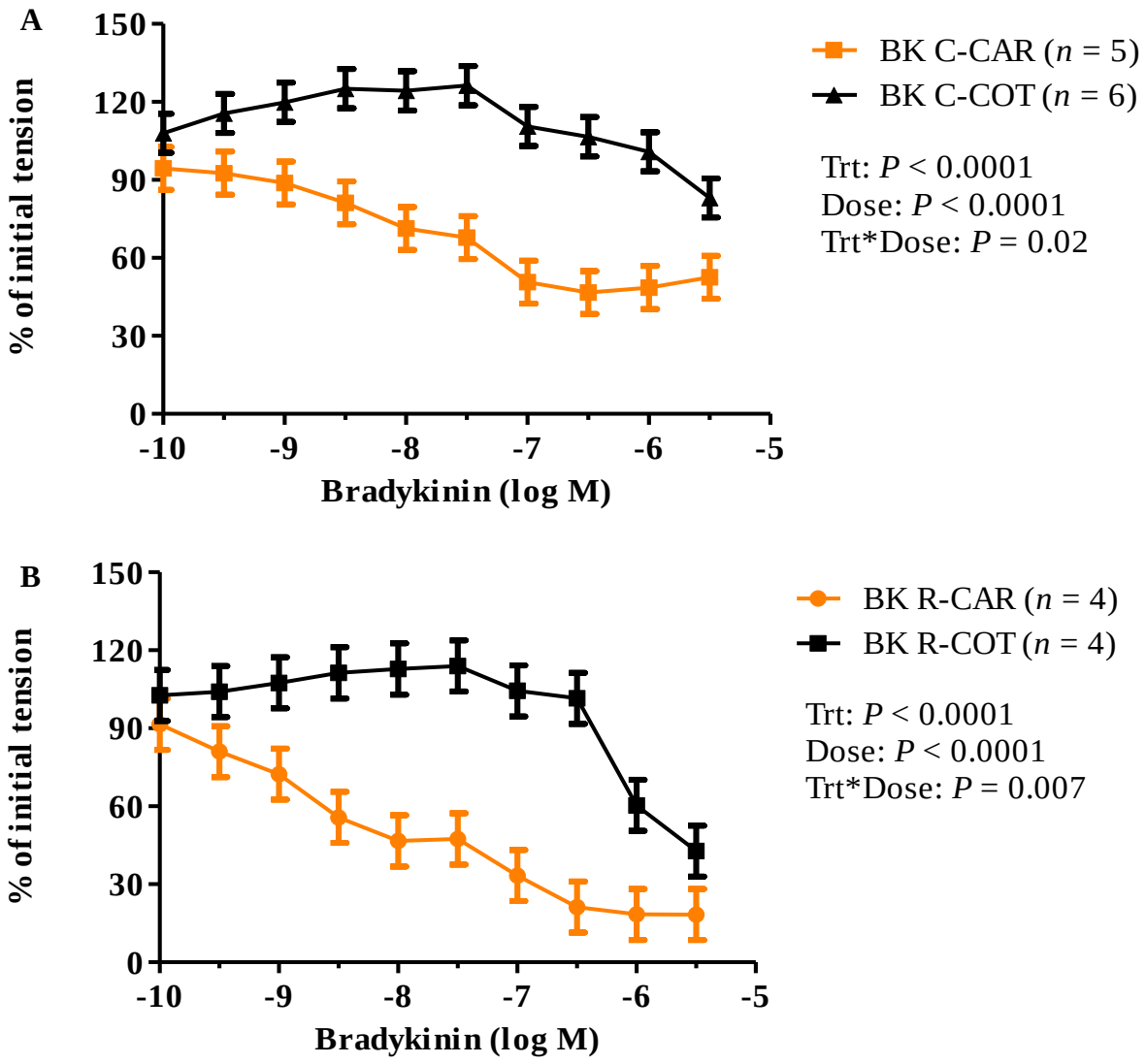

Figure 2.3. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in the caruncular (**CAR**) and cotyledonary (**COT**) arteries in cows receiving (**A**) 100% (**C**) of NRC requirements and (**B**) 60% (**R**) of NRC requirements. Diets were fed from d 30 to d 85 of gestation.

Discussion

Bradykinin, a nonapeptide kinin, is a stimulator of vascular permeability (Bhoola et al., 1992) and a potent vasodilator which enhances blood flow to the uterus during estrous cycle (Resnick et al., 1976) and the utero-placenta during gestation (Albertini et al., 1980). In addition, BK promotes angiogenesis, vascular permeability (Valdéz and Corthorn, 2011) and exerts its

physiological effects on the cardiovascular system by acting on the vascular endothelium (Hong, 1980; Cocks et al., 1985; Palmer et al., 1987; Gryglewski et al., 1986; Mombouli et al., 1996; Bas et al., 2007) via the the BK1 receptor and the BK2 receptor (BK2R) (Bhoola et al., 1992; Leeb-Lundberg et al., 2005), the latter being the predominant receptor type (Bas et al., 2007). The endothelial BK2R (Bhoola et al., 1992; Marceau et al., 1998; Christiansen et al., 2002; Marceau and Regoli, 2004; Souza et al., 2004; Moreau et al., 2005) promotes the release of endothelium derived vasodilatory mediators: nitric oxide (NO) (Furchgott and Zawadzki, 1980; Ignarro et al., 1981,1989; Bian and Murad, 2003; Furchgott, 1995; Moncada et al., 1991; Tanaka et al., 2004), prostacyclin (PGI_2) (Moncada et al., 1976; Coleman et al., 1994; Narumiya et al., 1999; Wise and Jones, 1996; Tanaka et al., 2004), and endothelium derived hyperpolarizing factors (EDHF(s)) (Nagao and Vanhoutte, 1993; McGuire et al., 2001; Busse et al., 2002; Suzuki, 2003; Triggle et al., 2003) for mediating vasorelaxation (Magness et al., 1993; Gainer et al., 1998).

The findings of this study reveal that global maternal nutrient restriction during early gestation (d 30 to d 85) increases the sensitivity of the CAR and the COT placental arteries in the R-NI cows to vasorelaxation compared with the C-NI cows in response to BK. These findings represent an important underlying mechanism by which dietary treatment alters placental vasoactivity which may impact uterine-umbilical blood flows and nutrient availability to the developing fetus. These conclusions are supported by the fact that the increase in sensitivity of the R-NI cows to BK induced relaxation allowed for compensatory placental growth resulting in enlarged placentas in cattle, as reported by our laboratory (Camacho et al., 2012). This increase in placental mass may have allowed for the tendency for increased fetal weight by increasing nutrient availability to the nutrient restricted cows (Camacho et al., 2012). These data are similar

to those of Lao and Wong (1996) who observed placental enlargement as an adaptive response induced as a result of lack of oxygen or nutrients and Lumey (1998), who also reported the delivery of heavier and enlarged placentas at term by nutritionally challenged human mothers during the first trimester of pregnancy. Similar to humans, compensatory placental growth was also observed in various models of undernourished ewes from early to mid-gestation (Owens et al., 1986; McCrabb et al., 1986, 1991; Faichney and White, 1987).

A successful pregnancy in all mammals is energetically costly and marks a 20-50% increase in the maternal metabolic rate (Brody, 1938; Ferrell et al., 1976; Ferrell and Jenkins, 1985; Robson et al., 1989; Stock and Metcalfe, 1994) primarily supporting the dramatic alterations in the maternal cardiovascular system and the utero-placental vascular bed (Rosenfeld and Fixler, 1977; Reynolds and Redmer, 1995; Magness, 1998) ensuring optimum fetal growth and development (Reynolds and Redmer, 1995; Reynolds et al., 2010). The placenta undergoes a variety of physiological changes to adapt to the various environmental stressors to drive fetal programming. Intrauterine growth restriction which is often caused by maternal under-nutrition during gestation can lead to stunted placental and fetal growth and development (Barker, 1997). Therefore, the development of potential therapeutics aimed at enhancing placental efficiency to promote nutrient delivery to the fetus would potentially be beneficial in overcoming compromised pregnancies (Reynolds et al., 2006, 2010).

In order to investigate the pathway responsible for BK induced relaxation, the CAR and the COT placental arteries were incubated in the presence of various inhibitors. The results of the present study show that the dietary treatment during early gestation influences vasorelaxation produced in response to BK which exerts its effects via the BK2R in the C and R cows in both CAR and COT placental arteries. Furthermore, BK_{Ca} channels are involved in mediating

relaxation in the CAR arteries; whereas, in the COT arteries, relaxation was enhanced upon incubation with IbTX in the C cows while no effect was observed in the R cows. In the current study, the C-IbTX and R-IbTX COT placental arteries relaxed despite blocking the BK_{Ca} channels. Since, the EDHF(s) are representative of a diverse group of chemical compounds comprising of L-citrulline, K^+ ion, H_2O_2, anandamide, and epoxyeicosatrienoic acids (Tanaka et al., 2004), it is quite possible that the observed relaxation in the COT placental arteries is likely due to an EDHF that is not inhibited by IbTX. This could possibly be a compensatory mechanism employed by the R COT placental arteries to ensure optimum nutrient delivery to the fetus. Therefore, further investigation of EDHF(s) is required which might provide a potential mechanism by which dietary treatment could potentiate BK induced relaxation in the COT placental arteries. In the presence of NLA, relaxation was inhibited in both, the CAR and the COT arteries pointing out the pivotal role of NO in mediating vasodilation in the placental arteries. Moreover, the E_{max} values of C-NLA and R-NLA arteries were significantly greater in the CAR and the COT arteries suggesting that the primary vasodilator is likely NO released from the endothelial cells in response to BK. Incubation with INDO inhibited vasorelaxation in the CAR placental arteries indicating the involvement of the PGI_2 pathway in mediating BK induced vasorelaxation.

The mRNA expression of the BK2R analyzed in the present study did not differ among the two treatment groups in both the CAR and the COT placental arteries, implying that while the receptor gene expression is not altered by nutritional status, enhanced BK activity must be augmented downstream from the predominant receptor type. These findings further emphasize the importance of vasoreactivity assays rather than relying on mRNA expression data as the sole determinant.

Lastly, it is of interest to note that maternal nutrient restriction during early gestation significantly enhances BK induced relaxation in the CAR arteries compared with the COT arteries from the C and the R cows. These results are important as they suggest that CAR vascular bed is more sensitive to BK induced vasodilation than the COT vascular bed during early gestation (d85) which is critical time for placental growth and development. This increased sensitivity could allow for an efficient nutrient delivery system to the fetus and may lay a foundation for the production of a healthy and viable offspring.

Taken together, the results of the present study support our hypothesis that global maternal nutrient restriction during early gestation in pregnant beef cows alters sensitivity of the CAR and the COT arteries to BK induced relaxation which is mediated by the BK2R and at least partly by the pathways mediating BK induced vasodilation in the placental arteries. Further investigations are required in the area of bovine maternal and fetal placental development during mid and late gestation and how potential therapeutics may impact compromised pregnancies in a cow.

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CHAPTER 3. INFLUENCE OF MATERNAL NUTRIENT RESTRICTION AND EARLY RESTRICTION FOLLOWED BY REALIMENTATION DURING MID-GESTATION ON PLACENTAL VASCULAR FUNCTION IN PREGNANT BEEF COWS

Abstract

We hypothesize that maternal nutrient restriction during early and mid-gestation and early restriction followed by realimentation would alter the caruncular (**CAR**) and cotyledonary (**COT**) placental artery sensitivity to bradykinin (**BK**) induced relaxation. Further, we hypothesized the placental arterial vasodilation is mediated at least partly through the bradykinin 2 receptor (**BK2R**) and its pathways which may be impacted by the maternal diet. To examine the effects of maternal nutrient restriction and realimentation on CAR and COT artery vasoactivity during mid-gestation, multiparous beef cows on d 30 of gestation were randomly assigned to receive 100% (d 30 to d 140) NRC requirements (**CC**; n = 6), 60% (d 30 to d 85) followed by 100% (d 85 to d 140) NRC requirements (**RC**; n = 5), and 60% NRC (d 85 to d 140) requirements (**RR**; n = 6). At d 140 of gestation cows were euthanized and arteries that terminated into the maternal (CAR artery) and fetal portions (COT artery) of the placentome were dissected to be used for in vitro vasoreactivity assays and mRNA expression. The endothelium-intact CAR arterial rings were loaded in an organ bath chamber and contracted with NE (10^{-6} M) and the concentration response curve (CRC) to BK was obtained. In addition, a BK CRC was obtained for rings which were incubated for 30 min with inhibitors to: 1) BK2R, icatibant acetate (**HOE 140**; 10^{-6} M); 2) large conductance Ca^{2+} activated K^{+} channels, iberiotoxin (**IbTX**; 10^{-7} M); 3) endogenous nitric oxide synthase, *N*(nitro)-L-arginine, (**NLA**; 10^{-5} M); and 4) prostacyclins (PGI_2), indomethacin (**INDO**; 10^{-5} M). Additionally, endothelium-intact COT arterial rings were contracted with U46619 (10^{-6} M) and the effect of BK and the above

mentioned inhibitors were studied with the exception of PGI_2. The CAR arterial rings with no inhibitor (**NI**) were influenced by the maternal diet and arteries from the RR cows were more sensitive ($P = 0.004$) to BK induced relaxation than the CC cows while they tended to relax more than the RC cows. Incubation with HOE 140 delayed relaxation ($P < 0.03$); however, the CAR arteries responded similarly at higher doses indicating the presence of an alternate mechanism or regulation of vasodilation partly via the BK1R in conjunction with the BK2R type. An effect of dietary treatment was observed in the presence of IbTX and NLA which inhibited relaxation ($P < 0.0001$) suggesting that BK_{Ca} channels and NO are involved in mediating relaxation in the CAR placental arteries. The CAR arteries from the RR cows had an effect of maternal diet upon incubation with INDO which inhibited relaxation ($P < 0.0001$) while no effect was observed in the CC ($P = 0.59$) and the RC ($P = 0.57$) arteries indicative of a compensatory mechanism employed by the CAR restricted arteries to overcome a longer period of nutrient restriction. In the COT placental arteries, the CC and the RR cows responded similarly and showed increased sensitivity ($P = 0.01$) to BK compared with the RC cows where relaxation was delayed. Arteries incubated with HOE 140 had a significant treatment by dose interaction which abolished ($P < 0.0001$) BK mediated relaxation. However, inhibition of BK_{Ca} channels failed to attenuate ($P > 0.06$) the BK response, indicating that BK_{Ca} channels may not be involved in BK responses in COT arteries. There was a treatment by dose interaction and NLA blocked ($P < 0.0001$) BK induced relaxation of COT arteries in all treatments. The mRNA expression of BK2R in placental arteries was not altered ($P \geq 0.50$) by the maternal diet during mid-gestation. Our findings reveal that the CAR and the COT placental arteries respond to BK through different mechanisms.

Key words: bradykinin, cow, placenta, vascular function

Introduction

Mid-gestation in the bovine is marked by maximal placental growth and differentiation (Barcroft and Barron, 1946; Metcalfe et al., 1988; Ferrell, 1989; Reynolds and Redmer, 1995; Bell et al., 1999) which continues to keep pace with the ever increasing fetal demands throughout gestation. Maternal nutrition during this crucial period of placental establishment has a significant impact on the fetal growth (Wallace, 1948; Wallace et al., 1999a; Godfrey and Barker, 2000). Numerous animal models investigating the effects of maternal diet in the ewes and cows have obtained variable results on the placental and fetal growth during mid-gestation in both species. Specific studies investigating the effects of realimentation in the bovine have produced contrasting results in the caruncular (**CAR**) and cotyledonary (**COT**) placental tissues (Vonnahme et al., 2007; Zhu et al., 2007).

As previously described (Reyaz, Chapter 2), our laboratory found that maternal nutrient restriction during early gestation resulted in increased sensitivity to bradykinin (**BK**) in the CAR and the COT placental arteries in the bovine. This increased sensitivity of placental arteries to BK may be a compensatory mechanism in the cow to overcome maternal nutrient restriction during early gestation. In order to further investigate the effect of dietary treatment during mid-gestation, vasoactivity assays were performed. We hypothesized that global maternal nutrient restriction during early and mid-gestation and early restriction followed by realimentation would alter placental arterial vascular function due to changes in placental arterial sensitivity to BK. Further, we also hypothesized that placental arterial vasodilation is mediated at least partly through the BK2R and that global maternal nutrient restriction may impact the pathways mediating BK induced vasodilation in the placental arteries. Thus, the objective of this study was to examine the effects of global maternal nutrient restriction and early restriction followed by

realimentation during mid-gestation on the mechanisms of BK induced vasorelaxation in the placental arteries of cow.

Materials and Methods

All procedures involving animals were approved by the North Dakota State University (NDSU) Animal Care and Use Committee (#A10001).

Animals, Diets, and Breeding

Animal breeding was similar as described in chapter 2; however, the durations of the applied dietary treatments were different. On d 30 of gestation multiparous beef cows were randomly assigned to receive 100% (d 30 to d 140) NRC requirements (**CC**; n = 6), 60% (d 30 to d 85) followed by 100% (d 85 to d 140) NRC requirements (**RC**; n = 5), and 60% NRC (d 85 to d 140) requirements (**RR**; n = 6). At d 140 of gestation cows were euthanized and arteries terminating into the maternal (caruncular, **CAR**) and the fetal (cotyledonary, **COT**) portions the placentome were dissected to be used for in vitro vasoreactivity assays and mRNA expression.

Tissue collection, experimental drug preparations, organ chamber studies, receptor quantification and data analysis methods are same as described in chapter 2.

Results

Caruncular Arterial Responses

In the CAR arteries, the treatment by dose interaction on the BK CRCs was not significant ($P = 0.99$) however, an effect of dietary treatment ($P = 0.004$) was observed (Figure 2.1A). The arteries from the RR-NI cows were more sensitive ($P < 0.001$) to BK ($10^{-10} – 10^{-6}$ M) induced vasodilation than arteries from the CC-NI cows. Moreover, there was a tendency for increased ($P = 0.088$) vasorelaxation to BK in the RR-NI arteries compared with the RC-NI

arteries while the RC-NI arteries did not differ ($P = 0.11$) from the CC-NI arteries. Maternal diet neither had an impact on the pD_2 values ($P = 0.63$; 8.67 ± 0.37 vs 8.62 ± 0.29 vs 8.98 ± 0.23 for CC-NI, RC-NI and RR-NI respectively) nor the E_{max} values ($P = 0.35$; 51.59 ± 11.94 vs 33.22 ± 11.94 vs 27.78 ± 10.89 for CC-NI, RC-NI and RR-NI respectively) for BK CRCs in CAR arteries with no inhibitor (NI).

The BK dose response curves in the presence of HOE 140 had a significant ($P = 0.0003$) treatment by dose interaction in CC-NI vs CC-HOE 140 arteries (Figure 2.2A). Arterial rings responded similarly until the 3×10^{-9} M dose of BK. Beginning at 10^{-8} M dose, CC-NI arterial rings were more sensitive until 3×10^{-7} M dose of BK while relaxation was delayed in CC-HOE 140 rings. However, at the highest doses of BK (10^{-6} M and 3×10^{-6} M), CC-HOE 140 rings showed higher sensitivity to BK induced relaxations compared with CC-NI rings. In the RC and RR aretries there was no treatment by dose interaction ($P \geq 0.21$) upon incubation with HOE 140. An overall effect of maternal nutrition was observed which delayed BK mediated vasodilation in the RC-HOE 140 vs RC-NI ($P < 0.0001$) arteries (Figure 3.3A) and RR-HOE 140 vs RR-NI ($P < 0.0001$) arteries (Figure 3.4A); however, at the highest dose (3×10^{-6} M) of BK, RC-HOE 140 and RR-HOE140 arteries responded to BK similarly to RC-NI and RR-NI arteries.

In the CAR placental arteries incubated with HOE 140, the pD_2 values were higher in the CC-HOE 140 ($P = 0.03$; 6.58 ± 0.45), RC-HOE-140 ($P = 0.03$; 6.82 ± 0.49), and RR-HOE 140 ($P < 0.01$; 7.02 ± 0.44) arteries compared with CC-NI (8.67 ± 0.52), RC-NI (8.61 ± 0.49), and RR-NI (8.97 ± 0.40) arteries. Further, The E_{max} value of the BK CRCs with HOE 140 was lower ($P = 0.03$) in the CC-HOE140 (22.48 ± 7.99) arteries vs CC-NI (51.59 ± 7.15) arteries while the E_{max} values in the RC ($P = 0.65$; 33.22 ± 16.96 vs 44.67 ± 16.96 for RC-NI and RC-HOE 140,

respectively) and RR (P = 0.77; 27.78 ± 9.99 vs 31.99 ± 9.99 for RR-NI and RR-HOE 140, respectively) cows were not influenced by the maternal diet.

For CAR placental arteries incubated with IbTX, the treatment by dose interaction was not significant in CC (P = 0.63), RC (P = 0.23), and RR (P = 0.85) arteries; however, an effect of maternal diet was observed which delayed relaxation in the CC-IbTX (P < 0.0001), RC-IbTX (P < 0.0001), and RR-IbTX (P < 0.0001) arteries compared with CC-NI (Figure 3.2B), RC-NI (Figure 3.3B), and RR-NI (Figure 3.4B) arteries.

The BK CRCs pD_2 values for CAR arteries with NI and CAR arteries incubated with IbTX were similar (P = 0.99; 8.67 ± 0.36 vs 8.67 ± 0.28 for CC-NI and CC-IbTX, respectively; P = 0.74; 8.62 ± 0.34 vs 8.45 ± 0.37 for RC-NI and RC-IbTX, respectively; P = 0.59; 8.98 ± 0.29 vs 8.73 ± 0.32 for RR-NI and RR-IbTX, respectively). In the CAR arteries incubated with IbTX, maternal diet led to a higher (P = 0.03) E_{max} value in the RC-IbTX (82.04 ± 13.39) vs the RC-NI (33.22 ± 13.39) arteries while the CC-IbTX (P = 0.06; 76.05 ± 7.87) and RR-IbTX (P = 0.07; 62.17 ± 11.97) had a tendency for a higher E_{max} than the CC-NI (51.59 ± 7.87) and RR-NI (27.17 ± 11.97) arteries.

The CAR placental arteries treated with NLA had a tendency for a treatment by dose interaction and a significant treatment effect (P < 0.0001) which inhibited relaxation but, this effect was only observed in the RC-NLA (P = 0.06) and RR-NLA (P = 0.09) arteries vs RC-NI (Figure 3.3C) and RR-NLA (Figure 3.4C) arteries. In the CC-NLA vs CC-NI arteries, the treatment by dose interaction was not significant (P = 0.32) but, an effect of dietary treatment (P < 0.0001) was observed (Figure 3.2C). The BK response curves for CC-NI and CC-NLA

followed a similar relaxation pattern until 3×10^{-9} M dose of BK, but after this dose, CC-NI arteries were more sensitive to BK mediated vasodilation.

The pD_2 values in CAR arteries incubated with NLA were not influenced ($P = 0.65$; 8.67 ± 0.17 vs 8.77 ± 0.13 for RR-NI and RR-NLA, respectively; $P = 0.22$; 8.62 ± 0.23 vs 8.15 ± 0.26 for RC-NI and RC-NLA, respectively; $P = 0.41$; 8.97 ± 0.34 vs 8.54 ± 0.38 for CC-NI and CC-NLA, respectively) by the maternal dietary treatment. Maternal diet did not impact the E_{max} of the BK CRCs incubated with NLA in CC arteries ($P = 0.12$; 51.59 ± 8.44 vs 71.30 ± 7.70 for CC-NI and CC-NLA, respectively); however, RC-NLA ($P = 0.04$; 78.83 ± 12.90) and RR-NLA ($P = 0.0002$; 80.44 ± 6.37) had greater E_{max} values compared with RC-NI (33.22 ± 12.90) and RR-NI (27.78 ± 6.37) arteries.

For CAR placental arteries incubated with INDO, neither the treatment by dose interaction ($P \geq 0.57$) nor an effect of dietary treatment ($P = 0.99$) was significant in CC-INDO vs CC-NI (Figure 3.2D) and RC-INDO vs RC-NI (Figure 3.3D) arteries. However, an effect of dose ($P < 0.0001$) was observed, where the CC and RC placental arteries responded similarly to BK in the presence and absence of INDO. However, in the RR arteries, an effect of maternal diet was observed in the presence of INDO which delayed relaxation ($P < 0.0001$) in the RR-INDO arteries compared with RR-NI (Figure 3.4D) arteries.

In the CAR arteries incubated with INDO, the pD_2 values were similar ($P = 0.88$; 8.67 ± 0.32 vs 8.60 ± 0.27 for CC-NI and CC-INDO, respectively; $P = 0.83$; 8.62 ± 0.28 vs 8.52 ± 0.31 for RC-NI and RR-INDO, respectively; $P = 0.47$; 8.98 ± 0.38 vs 8.47 ± 0.54 for RR-NI and RR-INDO, respectively). The BK dose response E_{max} value for CAR arteries in the presence of INDO was greater ($P < 0.01$) in RR-INDO (72.93 ± 10.37) vs RR-NI (27.78 ± 8.47) arteries

while no differences were observed on the E_{max} values in the CC (P = 0.93; 51.59 ± 8.27 vs 50.56 ± 8.27 for CC-NI and CC-INDO, respectively) and RC (P = 0.81; 33.22 ± 13.20 vs 28.28 ± 14.76 for RC-NI and RC-INDO, respectively) arteries.

Cotyledonary Arterial Responses

In the COT placental arteries, there was a tendency for a treatment by dose interaction (P = 0.06) and an overall effect (P = 0.01) of dietary treatment was observed when arteries were treated with BK (Figure 3.1B). There was a slight rightward shift in the RC-NI cows compared with the CC-NI (P = 0.008) and RR-NI (P < 0.02) cows, while the RR-NI did not differ from CC-NI arteries (P = 0.62).

Moreover, in the COT placental arteries with no inhibitor there was an overall effect (P < 0.03) of maternal nutrition on the pD_2 values. The RC-NI (6.60 ± 0.22) cows had a significantly higher (P < 0.01) pD_2 value compared with the RR-NI (7.53 ± 0.20) cows. In the RC arteries, the pD_2 value tended (P = 0.06) to be higher than the CC-NI (7.29 ± 0.25) cows while CC-NI cows did not significantly differ (P = 0.48) from the RR-NI cows. Maternal diet had no impact on the E_{max} values (P > 0.49; 21.09 ± 9.22 vs 23.77 ± 8.25 vs 29.63 ± 7.53 for CC-NI, RC-NI, and RR-NI, respectively) for the BK CRCs in COT placental arteries with NI.

The COT placental artery rings incubated with HOE 140 had a significant treatment by dose interaction and relaxation was completely blocked (i.e. 100%) in the CC-HOE 140 (P < 0.0001), RC-HOE 140 (P < 0.0001), and RR-HOE 140 (P < 0.0001) cows compared with CC-NI (Figure 3.5A), RC-NI (Figure 3.6A) and RR-NI (Figure 3.7A) arteries. The CC-HOE 140, RC-HOE 140, and RR-HOE 140 arteries responded similarly to BK until the 3×10^{-9} M, 3×10^{-8} M, 10^{-8} M doses of BK. After these doses, the arteries without an inhibitor were more sensitive to

BK induced vasodilation compared with the COT arteries incubated with HOE 140 for the remaining doses of BK.

In COT placental arteries incubated with HOE 140, the pD_2 values tended to be higher in the RC-NI (P = 0.06; 6.60 ± 0.27) vs RC-HOE 140 (7.41 ± 0.27) arteries while the BK response curve pD_2 values in the CC (P = 0.37; 7.29 ± 0.52 vs 7.96 ± 0.46 for CC-NI and CC-HOE 140, respectively) and RR (P = 0.41; 7.53 ± 0.42 vs 6.98 ± 0.42 for RR-NI and RR-HOE 140, respectively) aretries were not affected by the maternal diet. Additionally an effect of dietary treatment was observed on the E_{max} values in the presence of HOE 140. The CC-HOE 140 (P = 0.0004; 108.54 ± 9.15), RC-HOE 140 (P < 0.0001; 112.82 ± 6.15), and RR-HOE 140 (P = 0.0002; 103.00 ± 9.36) arteries had a significantly greater E_{max} values compared with CC-NI (21.09 ± 10.23), RC-NI (23.77 ± 6.15), and RR-NI (29.63 ± 9.36) arteries.

The BK CRCs did not have a significant ($P \geq 0.79$) treatment by dose interaction in the COT placental arteries treated with IbTX. The CC (P = 0.06) and RC (P = 0.09) cows had a tendency for an effect of dietary treatment while the RR cows were not influenced (P = 0.98) by the maternal diet. An effect of dose was observed in CC-IbTX, RC-IbTX, and RR-IbTX arteries which responded similarly (P < 0.0001) to BK compared with the CC-NI (Figure 3.5B), RC-NI (Figure 3.6B), and RR-NI (Figure 3.7B) arteries.

The BK dose response pD_2 values for COT arteries with no inhibitor and CAR arteries incubated with IbTX were similar (P = 0.73; 7.29 ± 0.31 vs 7.45 ± 0.31 for CC-NI and CC-IbTX, respectively; P = 0.83; 6.60 ± 0.18 vs 6.54 ± 0.18 for RC-NI and RC-IbTX, respectively; P = 0.35; 7.53 ± 0.19 vs 7.25 ± 0.20 for RR-NI and RR-IbTX, respectively). The COT artery rings E_{max} values in the presence of IbTX were not influenced by the maternal diet in CC (P = 0.79;

21.09 ± 9.84 vs 17.12 ± 9.84 for CC-NI and CC-IbTX, respectively), RC (P = 0.55; 23.77 ± 7.52 vs 17.12 ± 7.52 for RC-NI and RC-IbTX, respectively), and RR (P = 0.21; 29.63 ± 6.42 vs 16.66 ± 7.04 for RR-NI and RR-IbTX, respectively) cows.

In the COT placental artery, a significant treatment by dose interaction was observed when arterial rings were incubated with NLA. Regardless of maternal nutrition, relaxation was completely abolished (i.e. 100%) in the CC-NLA (P < 0.0001), RC-NLA (P < 0.0001), and RR-NLA (P < 0.0001) arteries compared with CC-NI (Figure 3.5C), RC-NI (Figure 3.6C), and RR-NI (Figure 3.7C) arteries.

In the COT arteries incubated with NLA, pD_2 value tended to be higher (P < 0.01) in the RC-NI (6.60 ± 0.31) arteries vs RC-NLA (8.13 ± 0.31) arteries while no differences were observed in the pD_2 values in the CC (P = 0.29; 7.29 ± 0.36 vs 7.82 ± 0.29 for CC-NI and CC-NLA, respectively) and RR (P = 0.15; 7.53 ± 0.19 vs 8.02 ± 0.24 for RR-NI and RR-NLA, respectively) cows. Moreover, the BK CRCs E_{max} value for COT arteries in the presence of NLA was significantly greater in the CC-NLA (P = 0.0001; 114.43 ± 8.75), RC-NLA (P < 0.0001; 112.12 ± 5.38), and RR-NLA (P < 0.0001, 124.10 ± 5.18) arteries compared with CC-NI (21.09 ± 10.71), RC-NI (23.77 ± 5.38), and RR-NI (29.63 ± 5.18) arteries.

Caruncular and Cotyledonary Arterial Responses to Bradykinin

The CRCs comparing the effects of BK in the CAR and COT placental arteries had a significant treatment by dose interaction in the CC-CAR vs CC-COT (P = 0.006) (Figure 3.8A), RC-CAR vs RC-COT (P < 0.0001) (Figure 3.8B) and RR-CAR vs RR-COT (P < 0.0001) (Figure 3.8C) arteries. The CC-CAR, RR-CAR were more sensitive to BK mediated vasodilation until the 3×10^{-8} M dose of BK and RC-CAR until 10^{-7} M dose of BK compared

with CC-COT, RR-COT, and RC-COT arteries. The CAR arteries reached a stable plateau at subsequent BK doses.

In the COT placental arteries relaxation was initially delayed in the CC-COT, RC-COT, and RR-COT arteries. However, in the CC-COT arteries sensitivity to BK was enhanced at the last two doses compared with CC-CAR rings, while the RC-COT and RR-COT responded similarly to RC-CAR and RR-CAR arteries beginning at 3×10^{-7} M and 10^{-7} M dose of BK.

Maternal nutrition influenced the pD_2 values in the COT placental arteries. The CC-COT ($P = 0.03$; 7.29 ± 0.29), RC-COT ($P < 0.0001$; 6.60 ± 0.17), and RR-COT ($P = 0.005$; 7.53 ± 0.28) had significantly higher pD_2 values compared with CC-CAR (8.67 ± 0.33) RC-CAR (8.62 ± 0.17) and RR-CAR (8.98 ± 0.28) arteries. Further, in the CC-COT arteries, the E_{max} values ($P = 0.07$) tended to be lower (21.09 ± 10.61) than the CC-CAR (51.59 ± 9.49) arteries while the BK response curves E_{max} values were not influenced in the RC ($P = 0.62$; 33.21 ± 12.98 vs 23.77 ± 12.98 for RC-CAR and RC-COT, respectively) and RR ($P = 0.87$; 27.78 ± 7.59 vs 29.63 ± 7.59 for RR-CAR and RR-COT, respectively) cows.

BK2R mRNA Expression

Maternal dietary treatment did not influence the mRNA expression of BK2R in the CAR ($P = 0.50$; 1.31, 1.49, 1.52 ± 0.14 arbitrary units for CC, RC, and RR, respectively) and the COT ($P = 0.58$; 1.39, 1.22, 1.34 ± 0.12 arbitrary units for CC, RC, and RR, respectively) placental arteries.

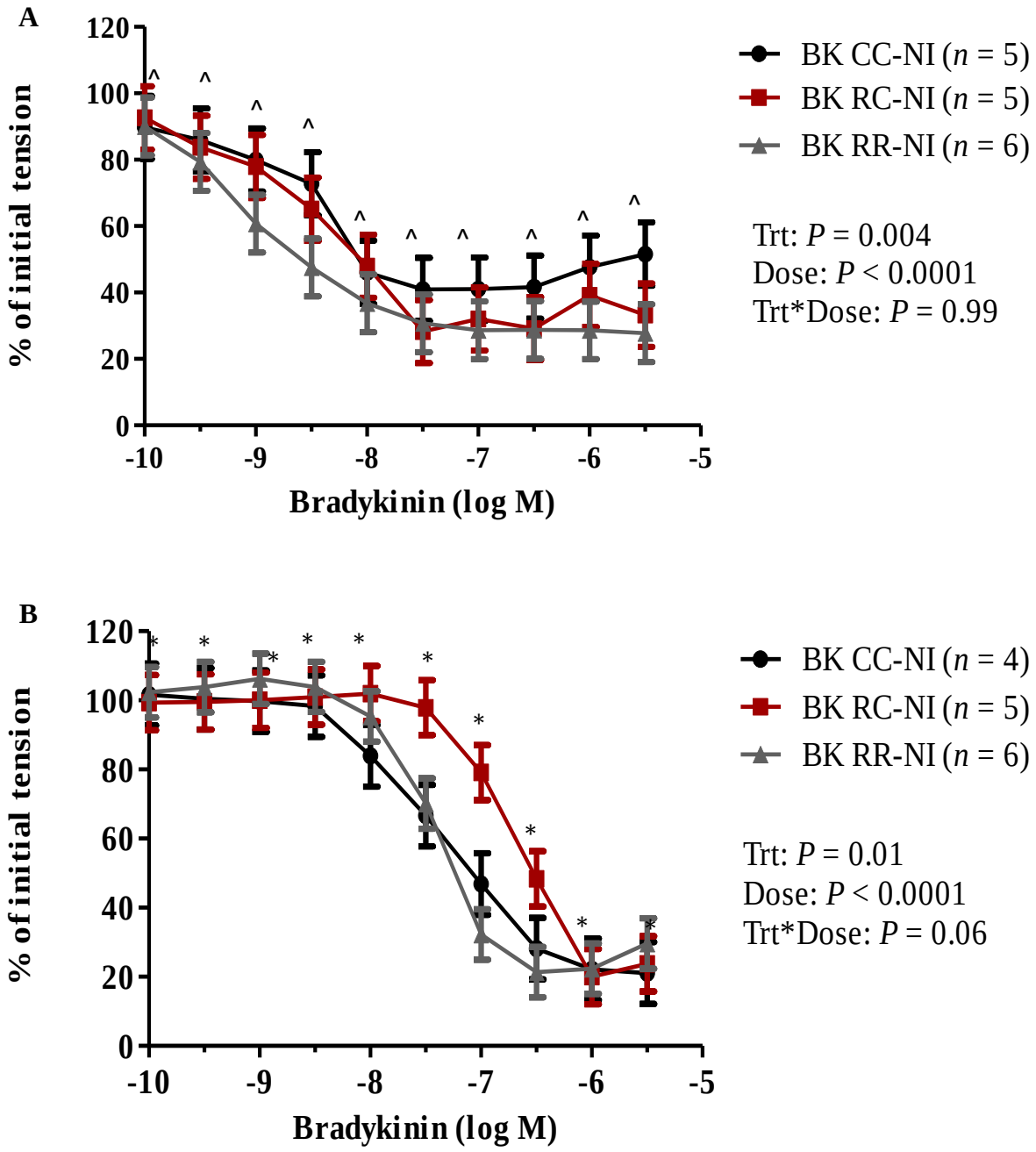

Figure 3.1. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in (**A**) caruncular (**CAR**) arteries and (**B**) cotyledonary (**COT**) arteries. Cows received 100% (**CC**) NRC requirements from d 30 to d 140, 60% (**RC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 140, and 60% (**RR**) NRC requirements from d 30 to d 140 of gestation. Figure compares BK concentration response curve obtained in the absence of any inhibitor (no inhibitor, **NI**) of each dietary treatment (CC vs RC vs RR). ^CC is statistically different from RR ($P < 0.001$). * RC is statistically different from CC ($P = 0.008$) and RR ($P < 0.02$).

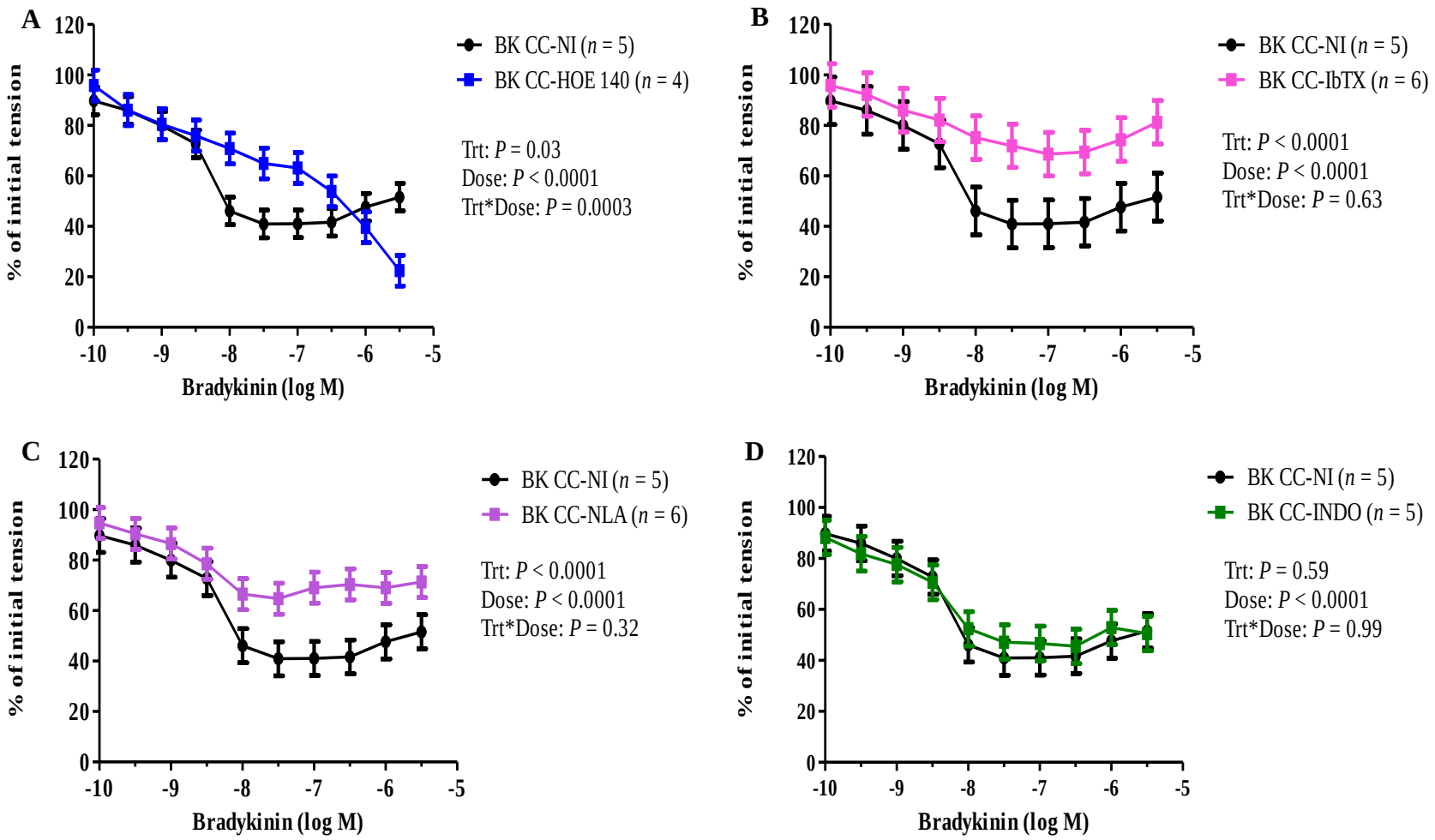

Figure 3.2. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in caruncular (**CAR**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**D**) after incubation with indomethacin (**INDO**). Cows received 100% (**CC**) NRC requirements from d 30 to d 140 of gestation.

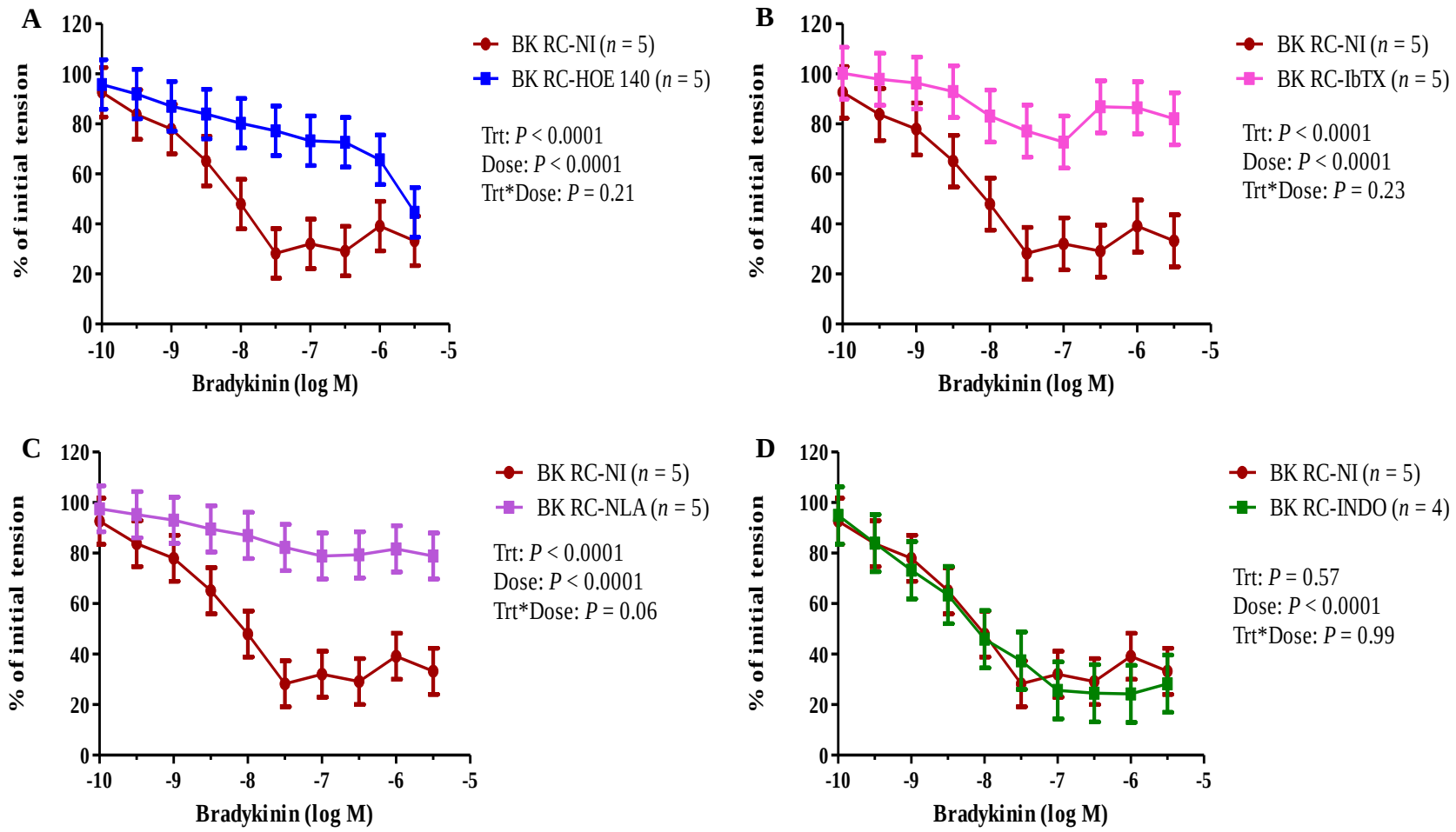

Figure 3.3. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in caruncular (**CAR**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**D**) after incubation with indomethacin (**INDO**). Cows received 60% (**RC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 140 of gestation.

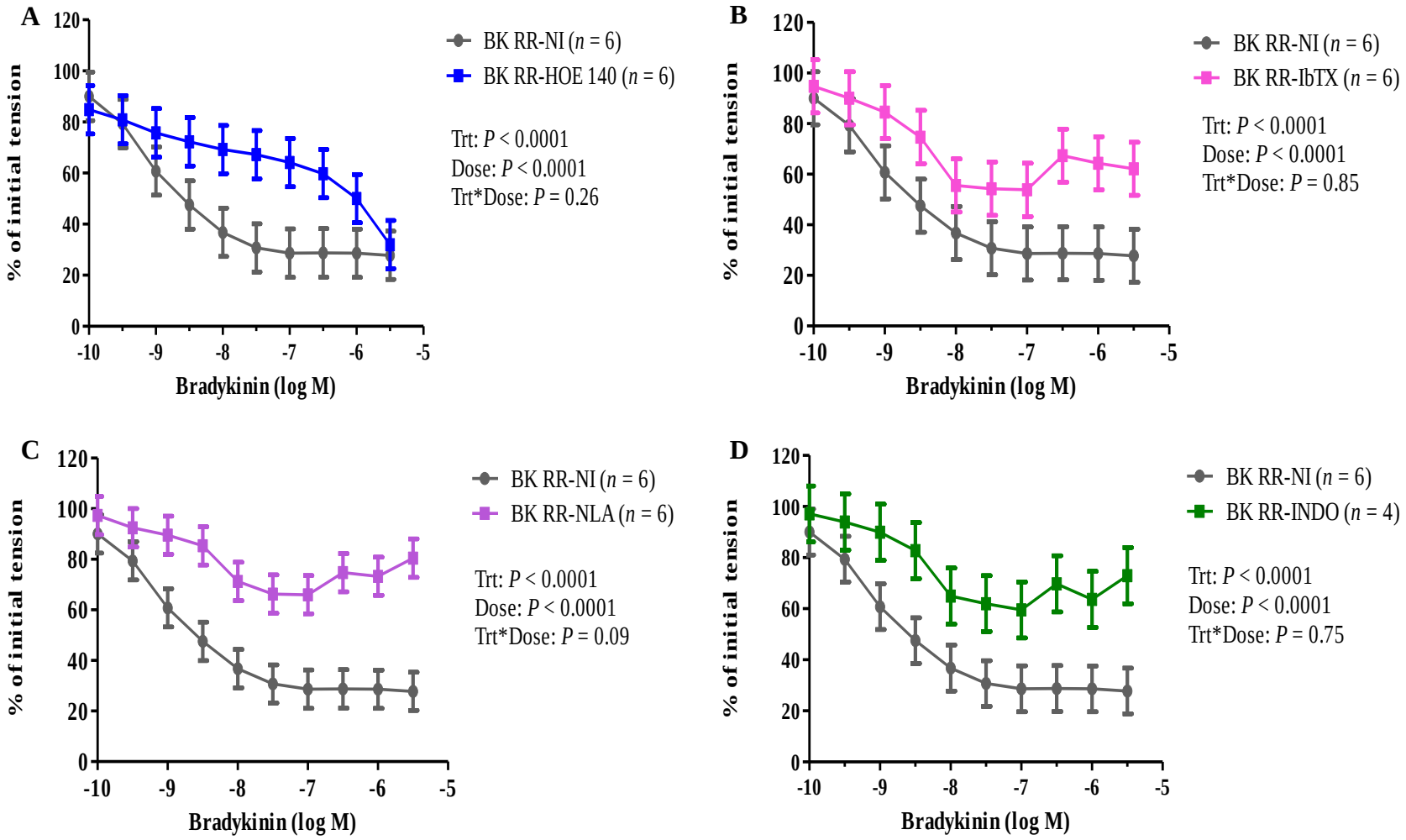

Figure 3.4. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in caruncular (**CAR**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**D**) after incubation with indomethacin (**INDO**). Cows received 60% (**RR**) NRC requirements from d 30 to d 140 of gestation.

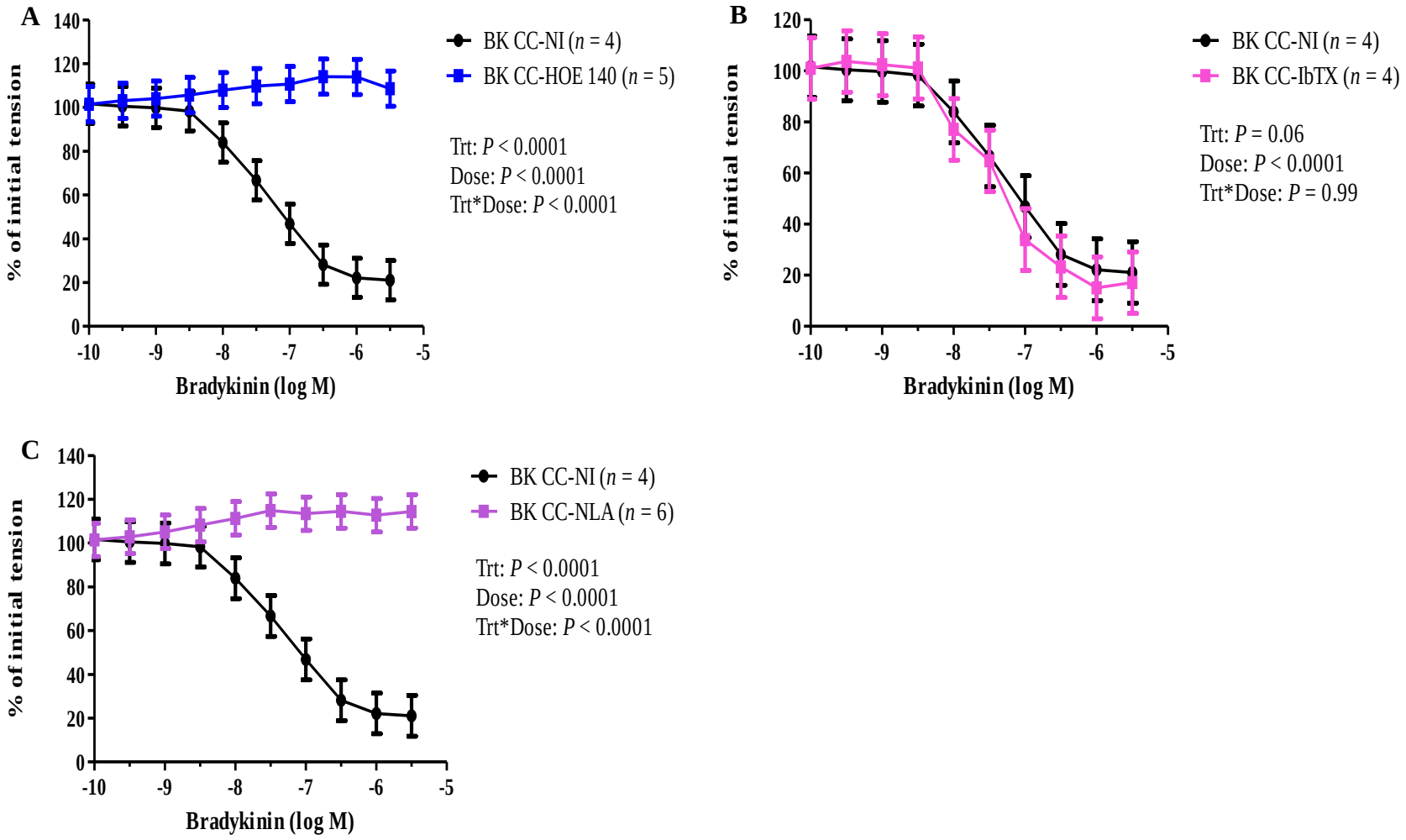

Figure 3.5. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), and (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**). Cows received 100% (**CC**) NRC requirements from d 30 to d 140 of gestation.

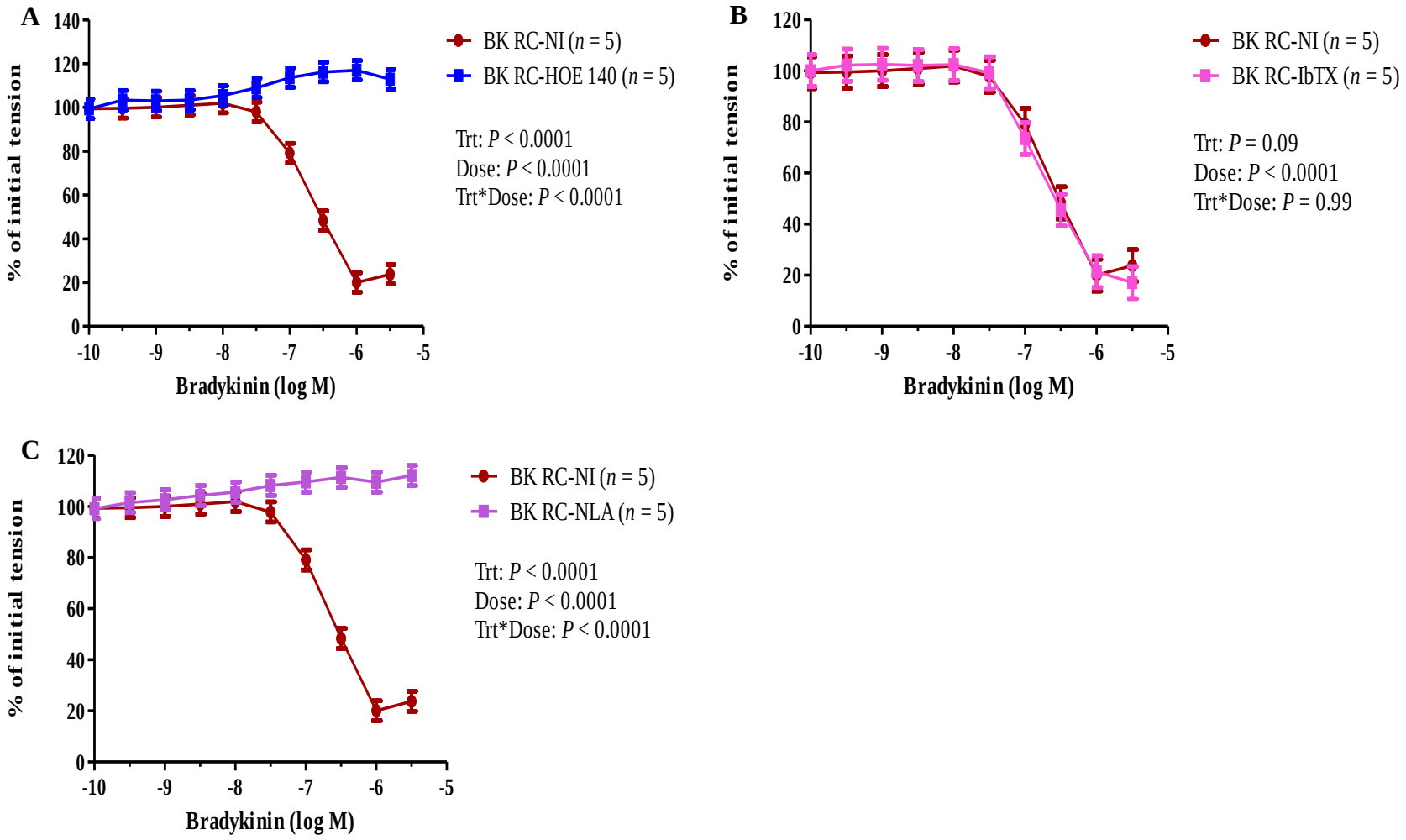

Figure 3.6. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), and (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**). Cows received 60% (**RC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 140 of gestation.

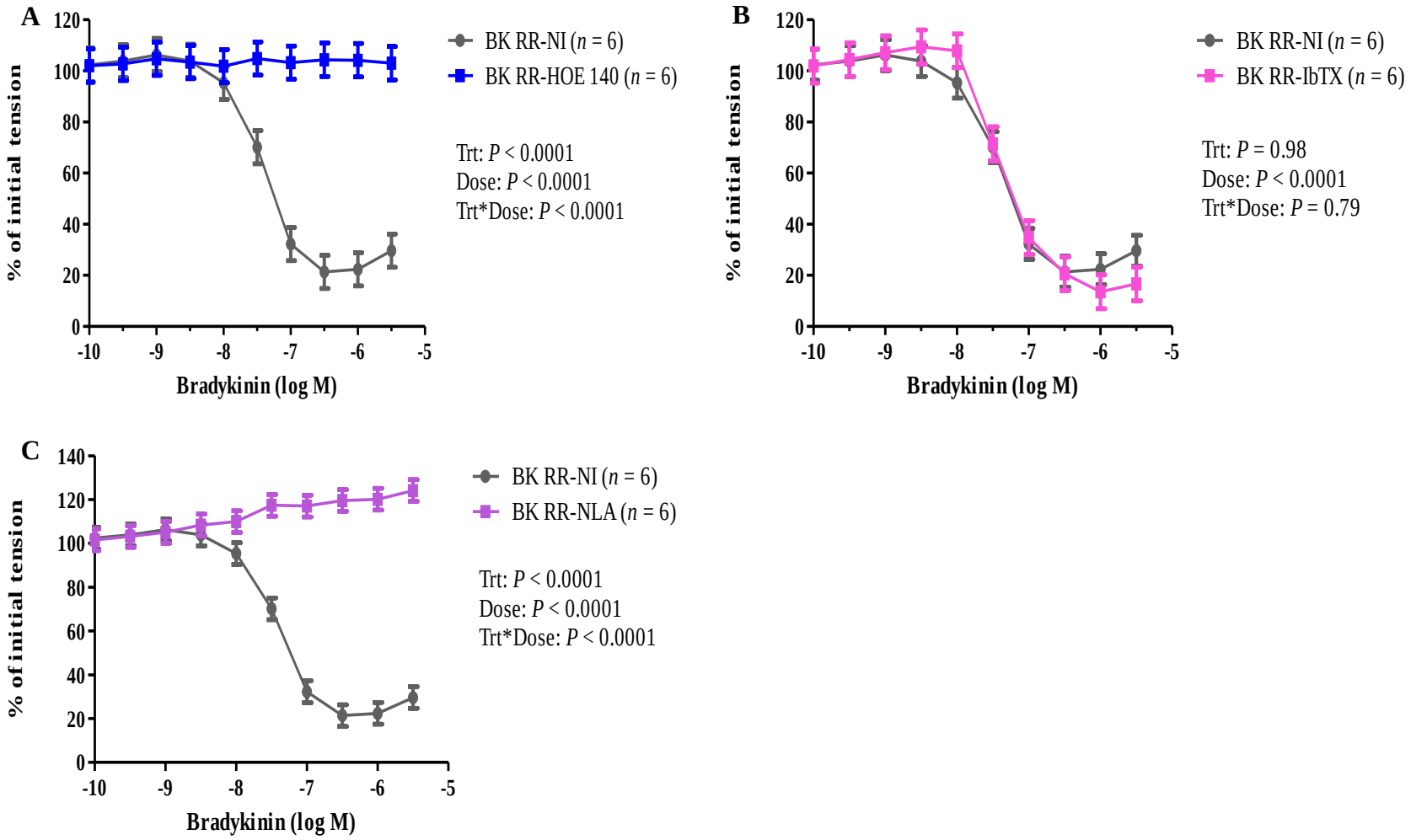

Figure 3.7. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), and (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**). Cows received 60% (**RR**) NRC requirements from d 30 to d 140 of gestation.

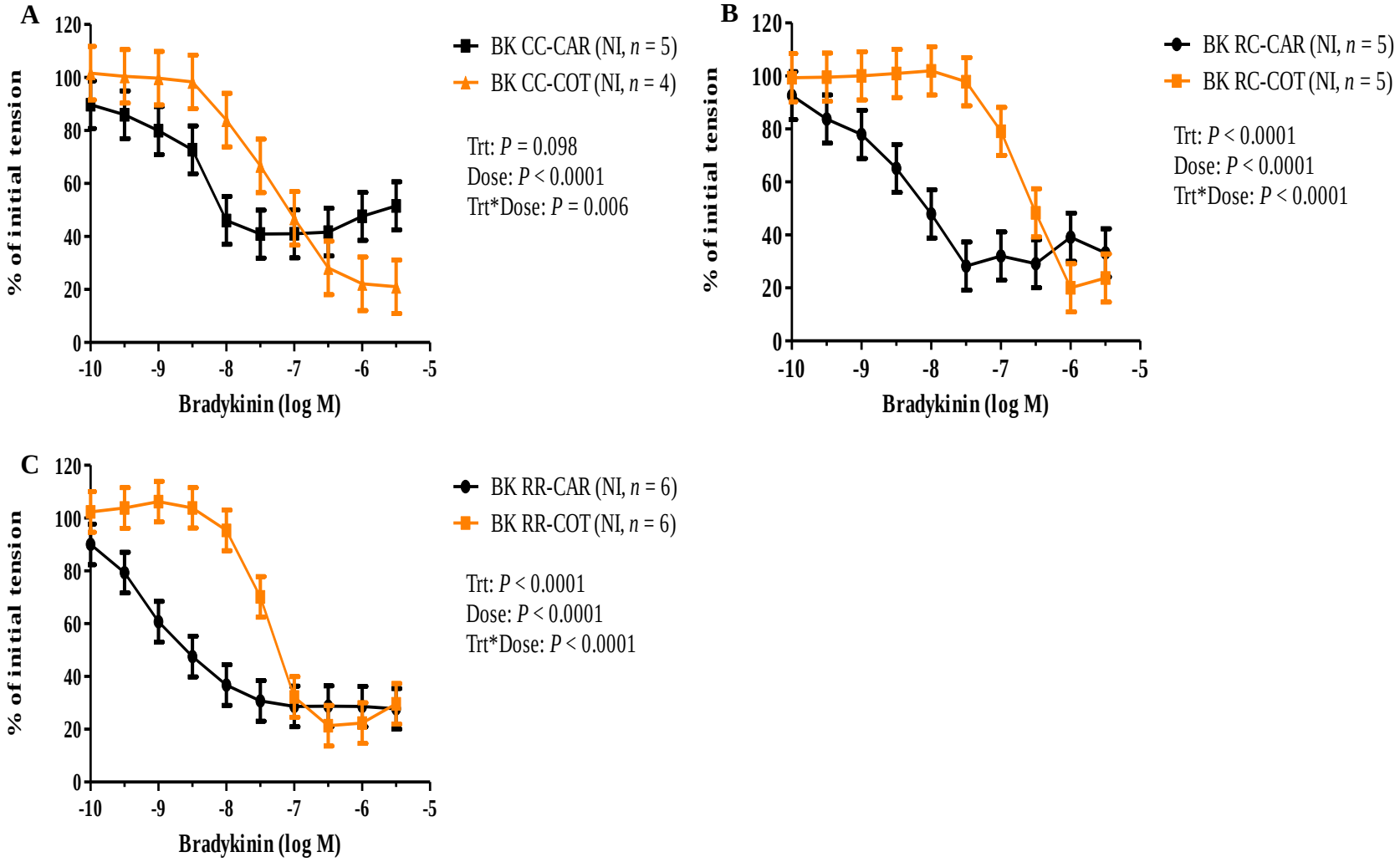

Figure 3.8. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in the caruncular (**CAR**) and cotyledonary (**COT**) arteries without an inhibitor (**NI**) in cows receiving (**A**) 100% (**CC**) NRC requirements from d 30 to d 140 of gestation, (**B**) 60% (**RC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 140 of gestation, and (**C**) 60% (**RR**) NRC requirements from d 30 to d 140 of gestation.

Discussion

While we observed a compensatory growth upon nutrient restriction during early gestation in both, the CAR and the COT portions of the placenta; it was interesting to note that at d 140 of gestation the two portions responded differently to restriction and restriction followed by realimentation. Global maternal nutrient restriction during early and mid-gestation (RR-NI; d 30 to d 140) increases the sensitivity of the CAR placental arteries to vasorelaxation in response BK when compared with the cows receiving 100% nutrient requirements (CC-NI) and produces a tendency for increased vasorelaxation than cows receiving a restricted diet (d 30 to d 85) during early gestation followed by realimentation (RC, d 85 to d 140). Our findings reveal that maternal nutrient restricition for a longer duration (from d 30 to d 140) is beneficial as it promotes vasodilation in the CAR placental arteries. On the other hand, in the COT placental arteries, the CC-NI and the RR-NI cows responded similarly and showed increased sensitivity to BK compared with the RC-NI cows where relaxation was delayed. Similar responses observed in the COT placental arteries in the RR-NI and the CC-NI cows might be a result of the nutrient restriction during early gestation which helps sustain vasodilation through mid-gestation in the RR-NI cows bringing placental vasoactivity similar to CC-NI cows in response to BK; while realimentation does not seem to have a beneficial effect on BK induced vasodilation in the RC-NI cows.

A successful pregnancy in all mammals is energetically costly and marks a 20-50% increase in the maternal metabolic rate (Brody, 1938; Ferrell et al., 1976; Ferrell and Jenkins, 1985; Robson et al., 1989; Stock and Metcalfe, 1994) primarily supporting the dramatic alterations in the maternal cardiovascular system and the utero-placental vascular bed (Rosenfeld and Fixler, 1977; Reynolds and Redmer, 1995; Magness, 1998) ensuring optimum fetal growth

and development (Reynolds and Redmer, 1995; Reynolds et al., 2010). The placenta undergoes a variety of physiological changes to adapt to the various environmental stressors to drive fetal programming. Intrauterine growth restriction which is often caused by maternal under-nutrition during gestation can lead to stunted placental and fetal growth and development (Barker, 1997). Therefore, the development of potential therapeutics aimed at enhancing placental efficiency to promote nutrient delivery to the fetus would potentially be beneficial in overcoming compromised pregnancies (Reynolds et al., 2006, 2010).

In order to investigate the pathway responsible for BK induced relaxation, the CAR and the COT placental arteries were incubated in the presence of various inhibitors. The results of the present study show that maternal nutrient restriction and restriction followed by realimentation influences vasorelaxation produced in response to BK; however, the mechanisms employed for mediating relaxation in the CAR and the COT placental arteries are different. The CAR placental arteries had a higher pD_2 value in the presence of HOE 140 which delayed relaxation at lower doses but, failed to inhibit relaxation at the highest dose indicative of an adaptive response or perhaps, regulation of vasodilation via the BK1R in conjuction with BK2R in the CAR placental arteries. Whereas, in the COT placental arteries relaxation was abolished and arteries incubated with HOE 140 had a significantly higher E_{max} value implying that the vasodilatory effects of BK are solely exerted via the BK2R in the COT placental arteries.

Furthermore, BK_{Ca} channels are involved in mediating relaxation in the CAR arteries; while, no effect was observed in the COT arteries upon incubation with IbTX. In the current study, the COT placental arteries relaxed despite blocking the BK_{Ca} channels. Since, the EDHF(s) are representative of a diverse group of chemical compounds comprising of L-citrulline, K^+ ion, H_2O_2, anandamide, and epoxyeicosatrienoic acids (Tanaka et al., 2004), it is

quite possible that the observed relaxations are likely due to an EDHF that is not inhibited by IbTX in the COT arteries. As gestation advances, the placenta is required to keep pace with the ever increasing fetal demands for growth and development. Thus, relaxation produced in the COT placental arteries might be an adaptive response to ensure optimum nutrient delivery to the fetus. Therefore, further investigation of EDHF(s) is required which might provide a potential mechanism responsible for mediating BK induced relaxation in COT placental arteries in the presence of IbTX.

In the presence of NLA, relaxation was inhibited in the CAR arteries and interestingly, E_{max} values of RC-NLA and RR-NLA arteries were significantly greater than RC-NI and RR-NI arteries suggesting that the role of NO is enhanced upon nutrient restriction and realimentation. Additional studies investigating the expression of eNOS across gestation would help explain the elevated role NO in the RC and the RR arteries. In the COT placental arteries relaxation was completely abolished and E_{max} values were greater in the presence of NLA which points out the pivotal role of NO in mediating vasodilation. The primary vasodilator is likely NO released from the endothelial cells in response to BK in the COT arteries.

Maternal diet had no effect on the CAR CC arteries and the RC arteries in the presence of INDO; however, relaxation was inhibited in the RR arteries which had a greater E_{max} value. This could possibly be a compensatory mechanism in the CAR arteries induced as a result of a longer nutrient restriction in the RR arteries to ensure optimum nutrient delivery to the developing fetus.

Similar to our findings at d 85 of gestation (Reyaz, Chapter 2) the mRNA expression of the BK2R analyzed in the present study did not differ across the three treatment groups in both the CAR and the COT placental arteries, implying that while the receptor gene expression is not

altered by nutritional status, enhanced BK activity must be augmented downstream from the predominant receptor type. It remains unknown how BK1R may change due to nutritional adaptation which might be a stress induced response to overcome nutrient restriction in the CAR placental arteries. These findings further emphasize the importance of vasoreactivity assays and perhaps a combination of the BK1R and the BK2R receptor inhibitors would help in better understanding of the specific role played by each receptor type across gestation.

Lastly, in the CAR placental arteries it is of interest to note that maternal nutrient restriction during early gestation followed by realimentation (RC-CAR) and a longer period of restriction (RR-CAR) significantly enhances BK induced relaxation however, their E_{max} values are similar with the COT arteries. While this held true for the RC and RR arteries, this effect was not observed in the CC-CAR arteries which had a tendency for a greater E_{max} compared with CC-COT arteries. Overall, the COT placental arteries had a greater pD_2 value suggesting that relaxation was delayed when compared with the CAR placental arteries. This is similar to our findings at d 85 of gestation; where we reported the CAR vascular bed to be more sensitive to BK induced vasodilation at lower doses of BK than the COT vascular bed. Since, early and mid-gestation is critical time for placental-fetal growth and development the increased sensitivity could allow for an efficient nutrient delivery system to the fetus.

Taken together, the results of the present study support our hypothesis that global maternal nutrient restriction or restriction followed by realimentation in pregnant beef cows alters sensitivity of the maternal and the fetal arteries to BK induced relaxation which is mediated by the BK2R and at least partly by the pathways mediating BK induced vasodilation in the placental arteries. Further investigations are required in the area of bovine maternal and fetal

placental development during late gestation and how potential therapeutics may impact compromised pregnancies in a cow.

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CHAPTER 4. INFLUENCE OF MATERNAL NUTRIENT RESTRICTION DURING EARLY AND MID-GESTATION FOLLOWED BY REALIMENTATION ON PLACENTAL VASCULAR FUNCTION IN LATE PREGNANT BEEF COWS

Abstract

We hypothesize that global maternal nutrient restriction during early and mid-gestation followed by realimentation would alter the caruncular (**CAR**) and cotyledonary (**COT**) placental artery sensitivity to bradykinin (**BK**) induced relaxation. Further, we hypothesized that placental arterial vasodilation is mediated at least partly through the bradykinin 2 receptor (**BK2R**) and its pathways which may be impacted by the maternal diet. To examine the effects of maternal nutrient restriction and realimentation on CAR and COT artery vasoactivity during mid-gestation, multiparous beef cows on d 30 of gestation were randomly assigned to receive 100% (d 30 to d 254) NRC requirements (**CCC**; n = 6), 60% (d 30 to d 85) followed by 100% (d 85 to d 254) NRC requirements (**RCC**; n = 5), and 60% (d 85 to d 140) followed by 100% (d 140 to d 254) NRC requirements (**RRC**; n = 6). At d 254 of gestation cows were euthanized and arteries that terminated into the maternal (CAR artery) and fetal portions (COT artery) of the placentome were dissected to be used for in vitro vasoreactivity assays and mRNA expression. The endothelium-intact CAR arterial rings were loaded in an organ bath chamber and contracted with NE (10^{-6} M) and the concentration response curve (CRC) to BK was obtained. In addition, a BK CRC was obtained for rings which were incubated for 30 min with inhibitors to: 1) BK2R, icatibant acetate (**HOE 140**; 10^{-6} M); 2) large conductance Ca^{2+} activated K^{+} channels, iberiotoxin (**IbTX**; 10^{-7} M); 3) endogenous nitric oxide synthase, *N*(nitro)-L-arginine, (**NLA**; 10^{-5} M); and 4) prostacyclins (PGI_2), indomethacin (**INDO**; 10^{-5} M). Additionally, endothelium-intact COT arterial rings were contracted with U46619 (10^{-6} M) and the effect of BK and the above

mentioned inhibitors were studied with the exception of PGI_2. The CAR arterial rings with no inhibitor (**NI**) were not influenced by the maternal diet and arteries responded similarly (P = 0.84) across treatments. Upon incubation with HOE 140, a treatment by dose interaction was observed in the RCC (P = 0.03) arteries while an effect of diet was observed in the CCC (P < 0.002) and the RRC (P < 0.0001) arteries which delayed relaxation at lower doses; however, the CAR arteries responded similarly at higher doses indicating the presence of an alternate mechanism or regulation of vasodilation partly via the BK1R in conjunction with the BK2R type. An effect of dietary treatment was observed in the three treatment groups in the presence of IbTX which inhibited relaxation (P < 0.0001) suggesting that BK_{Ca} channels are involved in mediating relaxation in the CAR placental arteries. There was a treatment by dose interaction upon incubation with NLA in the RCC (P < 0.0001) and the RRC (P = 0.05) cows while an effect of diet was observed in the CCC (P < 0.0001) arteries which blocked relaxation. There was also an effect of maternal diet in the RCC (P < 0.001) and RRC (P < 0.01) arteries in the presence of INDO which delayed relaxation at lower doses while no effect was observed in the arteries from CCC (P = 0.71) cows. The NI COT arterial rings were not influenced by the maternal diet and arteries responded similarly (P = 0.10) across treatments. A treatment by dose interaction was observed in the CCC (P = 0.002) and the RCC (P < 0.0001) arteries in the presence of HOE 140 and relaxation was inhibited while an effect of diet was observed in the RRC (P < 0.04) arteries. Inhibition of BK_{Ca} channels failed to attenuate (P > 0.19) the BK response across treatments, indicating that BK_{Ca} channels may not be involved in BK responses in COT arteries. There was a treatment by dose interaction and NLA blocked (P < 0.0008) BK induced relaxation in all treatments. The mRNA expression of BK2R was not altered ($P \geq 0.48$) by the maternal diet during late gestation. It appears that placental arteries have completely

adapted after the two durations of nutrient restriction followed by realimentation and are responding similarly to the control arteries. Despite this, there may be different durations of restriction which may program the placenta to compensate for the loss of nutrients by vasodilation thorugh different BK mediated mechanisms.

Key words: bradykinin, cow, placenta, vascular function

Introduction

By late gestation, the primary function of the placenta is to support the exponential growth of the fetus during the last half of gestation (Ferrell, 1989; Metcalfe, 1998; Reynolds and Redmer, 1995). Several models of underfed ewes during late gestation have found to hinder the fetal growth trajectory (Mellor, 1983; Robinson, 1983; Vincent et al., 1985; Parr et al., 1986) and transplacental exchanges. Limited studies investigating the effects of maternal nutrient restriction followed by realimentation in the bovine have reported an increase in the CAR tissue which might result in enhanced placental efficiency in the nutrient restricted cows (Vonnahme et al., 2007; Zhu et al., 2007).

While we observed a compensatory growth upon nutrient restriction during early gestation (d 85) in both, the caruncular (CAR) and the cotyledonary (COT) portions of the placenta; it was interesting to note that at d 140 of gestation the two portions responded differently to restriction and restriction followed by realimentation. In order to further investigate the effect of dietary treatment during late gestation, vasoactivity assays were performed on placental arteries. We hypothesized that global maternal nutrient restriction during early and mid-gestation followed by realimentation in pregnant beef cows would alter placental arterial vascular function due to changes in placental arterial sensitivity to BK. Further, we also

hypothesized that placental arterial vasodilation is mediated at least partly through the BK2R and that global maternal nutrient restriction may impact the pathways mediating BK induced vasodilation in the placental arteries. Thus, the objective of this study was to examine the effects of global maternal nutrient restriction during early and mid-gestation followed by realimentation on the mechanisms of BK induced vasorelaxation in the placental arteries of cow.

Materials and Methods

All procedures involving animals were approved by the North Dakota State University (NDSU) Animal Care and Use Committee (#A10001).

Animals, Diets, and Breeding

Animal breeding was similar as described in chapter 2; however, the durations of the applied dietary treatments were different. On d 30 of gestation multiparous beef cows were randomly assigned to receive 100% (d 30 to d 254) NRC requirements (**CCC**; n = 6), 60% (d 30 to d 85) followed by 100% (d 85 to d 254) NRC requirements (**RCC**; n = 5), and 60% (d 85 to d 140) followed by 100% (d 140 to d 254) NRC requirements (**RRC**; n = 6). At d 254 of gestation cows were euthanized and arteries terminating into the maternal (caruncular, **CAR**) and the fetal (cotyledonary, **COT**) portions the placentome were dissected to be used for in vitro vasoreactivity assays and mRNA expression.

Tissue collection, experimental drug preparations, organ chamber studies, receptor quantification and data analysis methods are same as described in chapter 2.

Results

Caruncular Arterial Responses

In the CAR arteries there was no interaction ($P = 0.81$) neither an effect of treatment ($P = 0.84$) was observed on the BK CRCs with no inhibitor (NI) (Figure 4.1A). There was an effect of dose ($P < 0.0001$) where CCC-NI, RCC-NI, and RRC-NI arteries responded similarly to BK. Maternal diet neither had a impact on the pD_2 values ($P = 0.52$; 8.48 ± 0.37 vs 8.81 ± 0.37 vs 9.05 ± 0.33 for CCC-NI, RCC-NI, and RRC-NI respectively) nor the E_{max} values ($P = 0.97$; 38.33 ± 12.76 vs 41.06 ± 13.98 vs 42.53 ± 13.98 for CCC-NI, RCC-NI, and RRC-NI respectively) for BK CRCs in CAR arteries with NI.

The CCC-NI vs CCC-HOE 140 arteries did not have a significant ($P = 0.99$) treatment by dose interaction in the presence of HOE 140 but, an effect of maternal nutrition ($P = 0.002$) was observed (Figure 4.2A). The arteries depicted a similar pattern of relaxation which was not inhibited upon incubation with HOE 140. The BK dose response curves in the presence of HOE 140 had a significant ($P = 0.03$) treatment by dose interaction in RCC-NI vs RCC-HOE 140 arteries (Figure 4.3A). Arterial rings responded similarly until the 10^{-9} M dose of BK. Beginning at 3×10^{-9} M dose, RCC-NI arterial rings were more sensitive to vasodilation until 10^{-6} M dose of BK while relaxation was delayed in RCC-HOE 140 rings. However, at the highest doses of BK (3×10^{-6} M) RCC-NI and RCC-HOE 140 arteries responded similarly to BK. In the RRC-NI vs RRC-HOE 140 arteries there was no treatment by dose interaction ($P = 0.71$) upon incubation with HOE 140. An overall effect of maternal nutrition ($P < 0.0001$) was observed which delayed BK mediated vasodilation in the RRC-HOE 140 vs RRC-NI arteries (Figure 4.4A); however, at the highest dose (3×10^{-6} M) of BK the arteries responded similarly.

In the CAR placental arteries incubated with HOE 140 the pD_2 values were higher (P = 0.02) in the RRC-HOE 140 (7.05 ± 0.48) arteries vs RRC-NI (9.05 ± 0.48) arteries. Maternal diet did not impact the pD_2 values in the CCC (P = 0.48; 8.46 ± 0.59 vs 7.78 ± 0.68 for CCC-NI and CCC-HOE 140, respectively) and RCC cows (P = 0.79; 8.81 ± 0.29 vs 8.92 ± 0.29 for RCC-NI and RCC-HOE 140, respectively). Further, the E_{max} values of the BK CRCs with HOE 140 were not influenced by the maternal diet in the CCC (P = 0.56; 38.33 ± 10.90 vs 48.24 ± 12.04 for CCC-NI and CCC-HOE 140, respectively), RCC (P = 0.64; 41.06 ± 11.51 vs 49.46 ± 12.87 for RCC-NI and RCC-HOE 140, respectively), and RRC (P = 0.82; 42.53 ± 11.82 vs 46.55 ± 11.82 for RRC-NI and RRC-HOE 140, respectively) arteries for HOE 140.

In the CAR placental arteries incubated with IbTX, a tendency for a treatment by dose interaction (P = 0.06) was observed in the RCC arteries and an effect of maternal diet was observed in the CCC-IbTX (P < 0.0001), RCC-IbTX (P < 0.0001), and RRC-IbTX (P < 0.0001) arteries which inhibited relaxation compared with CCC-NI (Figure 4.2B), RCC-NI (Figure 4.3B), and RRC-NI (Figure 4.4B) arteries.

The BK CRCs pD_2 values for CAR placental arteries with IbTX tended to be higher (P = 0.06) in the RRC- IbTX (7.36 ± 0.59) arteries compared with RRC-NI (9.05 ± 0.46) arteries while no effect of maternal diet was observed in the CCC (P = 0.74; 8.48 ± 0.59 vs 8.12 ± 0.83 for CCC-NI and CCC-IbTX, respectively) and the RCC (P = 0.10; 8.81 ± 0.23 vs 7.59 ± 0.47 for RCC-NI and RCC-IbTX, respectively) arteries. In the CAR arteries incubated with IbTX, CCC-IbTX (P < 0.01; 96.22 ± 13.11) and RRC-IbTX (P = 0.01; 96.94 ± 11.60) arteries had a greater E_{max} value compared with CCC-NI (38.33 ± 10.70) and RRC-NI (42.53 ± 11.60) arteries while RCC-IbTX (P = 0.08; 85.18 ± 16.07) arteries had a tendency for a greater E_{max} value than the RCC-NI (41.06 ± 14.37) arteries.

The CAR placental arteries treated with NLA had a significant treatment by dose interaction which blocked relaxation however, this effect was only observed in the RCC-NLA ($P < 0.0001$) and RRC-NLA ($P = 0.05$) arteries vs RCC-NI (Figure 4.3C) and RRC-NLA (Figure 4.4C) arteries. In the CCC-NLA vs CCC-NI arteries, the treatment by dose interaction was not significant ($P = 0.83$) but, an effect of dietary treatment ($P < 0.0001$) was observed (Figure 4.2C) which inhibited BK mediated relaxation in the presence of NLA.

The pD_2 values in CAR arteries incubated with NLA were not influenced ($P = 0.59$; 8.48 ± 0.56 vs 8.07 ± 0.46 for CCC-NI and CCC-NLA, respectively; $P = 0.18$; 8.81 ± 0.39 vs 7.71 ± 0.55 for RCC-NI and RCC-NLA, respectively; $P = 0.18$; 9.05 ± 0.57 vs 7.94 ± 0.52 for RRC-NI and RRC-NLA, respectively) by the maternal dietary treatment. The E_{max} of the BK CRCs incubated with NLA was greater than the arteries with NI ($P < 0.01$; 38.33 ± 10.83 vs 88.42 ± 10.83 for CCC-NI and CCC-NLA, respectively; $P < 0.01$; 41.06 ± 11.47 vs 105.85 ± 12.83 for RCC-NI and RCC-NLA, respectively; $P < 0.003$; 42.53 ± 9.04 vs 93.00 ± 8.25 for RRC-NI and RRC-NLA, respectively).

For CAR placental arteries incubated with INDO, neither the treatment by dose interaction ($P = 0.98$) nor an effect of dietary treatment ($P = 0.71$) was significant in CCC-INDO vs CCC-NI arteries (Figure 4.2D) and relaxation was not inhibited upon incubation with INDO. The treatment by dose interaction was not significant however, an effect of treatment was observed in RCC ($P = 0.001$) and RRC ($P = 0.01$) arteries. The RCC-NI and RCC-INDO (Figure 4.3D) arteries responded similarly until 3×10^{-9} M dose of BK. Beginning at the 10^{-8} M dose, relaxation was delayed in RCC-INDO arteries however, at the last dose (3×10^{-6} M) of BK, arteries responded similarly. Similar patterns of relaxation were observed in the RRC-INDO arteries vs the RRC-NI arteries (Figure 4.4D).

In the CAR arteries incubated with INDO, the pD_2 values were similar (P = 0.38; 8.48 ± 0.47 vs 9.11 ± 0.47 for CCC-NI and CCC-INDO, respectively; P = 0.14; 8.80 ± 0.55 vs 7.48 ± 0.55 for RCC-NI and RCC-INDO, respectively; P = 0.33; 9.05 ± 0.37 vs 8.50 ± 0.37 for RRC-NI and RRC-INDO, respectively) across the three treatment groups. Moreover, no differences were observed on the E_{max} BK CRCs (P = 0.62; 38.33 ± 12.75 vs 48.04 ± 13.96 for CCC-NI and CCC-INDO, respectively; P = 0.97; 41.06 ± 12.62 vs 40.29 ± 14.11 for RCC-NI and RCC-INDO, respectively; P = 0.85; 42.53 ± 12.62 vs 46.08 ± 12.62 for RRC-NI and RRC-INDO, respectively) in the CAR placental arteries.

Cotyledonary Arterial Responses

In the COT placental arteries neither the treatment by dose interaction (P = 0.99) nor an effect of dietary treatment (P = 0.10) was significant on the BK CRCs with NI (Figure 4.1B). There was an effect of dose (P < 0.0001) and CCC-NI, RCC-NI, and RRC-NI arteries responded similarly to BK. Maternal diet did not impact the pD_2 values (P = 0.31; 6.54 ± 0.72 vs 6.19 ± 0.24 vs 6.71 ± 0.56 for CCC-NI, RCC-NI and RRC-NI respectively) and the E_{max} values (P = 0.82; 53.67 ± 7.78 vs 53.69 ± 8.70 vs 59.17 ± 7.78 for CCC-NI, RCC-NI, and RRC-NI respectively) for BK CRCs in CAR arteries with NI.

The COT placental artery rings incubated with HOE 140 had a significant treatment by dose interaction in the CCC (P = 0.002) (Figure 4.5A) and RCC (P < 0.0001) (Figure 4.6A) arteries. The CCC-HOE 140 and RCC-HOE 140 aretries responded similarly to CCC-NI and RCC-NI arteries until 10^{-7} M and 3 ×10^{-7} M dose of BK after which relaxation was inhibited at susbsequent BK doses. In the RRC cows the treatment by dose interaction was not significant (P = 0.59); however, an effect of treatment (P = 0.04) was observed. The RRC-NI and RRC-HOE

140 arteries responded similarly to BK until the 3×10^{-7} M dose, after which relaxation was delayed in the RRC-HOE 140 arteries (Figure 4.7A).

In the COT placental arteries incubated with HOE 140, the pD_2 values were similar (P = 0.88; 6.54 ± 0.47 vs 6.48 ± 0.33 for CCC-NI and CCC-HOE 140, respectively; P = 0.25; 6.19 ± 0.71 vs 7.48 ± 0.71 for RCC-NI and RCC-HOE 140, respectively; P = 0.92; 6.71 ± 0.32 vs 6.75 ± 0.29 for RRC-NI and RRC-HOE 140, respectively) to arteries with NI. An effect of dietary treatment was observed on the E_{max} values in the presence of HOE 140 in the CCC and the RCC arteries. The CCC-HOE 140 (P = 0.03; 80.72 ± 7.3) and RCC-HOE 140 (P < 0.01; 88.02 ± 6.23) arteries had a greater E_{max} value compared with CCC-NI (52.67 ± 7.99) and RCC-NI (53.69 ± 6.23) arteries while the E_{max} BK CRCs for RRC cows were not different (P = 0.32; 59.17 ± 7.99 vs 70.39 ± 7.29 for RRC-NI and RRC-HOE 140, respectively).

The BK CRCs in the presence of IbTX had a significant treatment by dose interaction (P = 0.01); however this effect was only observed in the RCC-NI vs RCC-IbTX arteries (Figure 4.6B). In the CCC and the RRC arteries neither the treatment by dose interaction ($P \geq 0.82$) nor an effect of dietary treatment ($P \geq 0.35$) was significant. There was an effect of dose (P < 0.0001) and CCC-NI and RRC-NI arteries responded similarly to CCC-IbTX (Figure 4.5B) and RRC-IbTX (Figure 4.7B) arteries.

The BK CRCs pD_2 values for COT arteries with NI and COT arteries incubated with IbTX were similar (P = 0.96; 6.54 ± 0.10 vs 6.55 ± 0.09 for CCC-NI and CCC-IbTX, respectively; P = 0.81; 6.19 ± 0.18 vs 6.25 ± 0.16 for RCC-NI and RCC-IbTX, respectively; P = 0.72; 6.71 ± 0.41 vs 6.91 ± 0.38 for RRC-NI and RRC-IbTX, respectively). The COT artery rings E_{max} values in the presence of IbTX were not influenced by the maternal diet in CCC (P =

0.44; 52.67 ± 10.94 vs 65.29 ± 10.94 for CCC-NI and CCC-IbTX, respectively), RCC ($P = 0.11$; 53.69 ± 6.53 vs 69.97 ± 5.84 for RCC-NI and RCC-IbTX, respectively), and RRC ($P = 0.82$; 59.17 ± 6.14 vs 61.09 ± 5.61 for RRC-NI and RRC-IbTX, respectively) cows.

Upon incubation with NLA, a significant treatment by dose interaction was observed in CCC-NLA ($P = 0.0006$), RCC-NLA ($P < 0.0001$), and RRC-NLA ($P = 0.0008$) arteries compared with CCC-NI (Figure 4.5C), RCC-NI (Figure 4.6C), and RRC-NI (Figure 4.7C) arteries. The COT arteries from all cows responded similarly to BK until the 10^{-7} M dose of BK, after which the arteries with NI were more sensitive to BK while relaxation was inhibited in the NLA incubated arteries.

In the COT placental arteries incubated with NLA, pD_2 values were not affected by the maternal diet ($P = 0.47$; 6.54 ± 0.16 vs 6.38 ± 0.14 for CCC-NI and CCC-NLA, respectively; $P = 0.80$; 6.19 ± 0.25 vs 6.09 ± 0.29 for RCC-NI and RCC-NLA, respectively; $P = 0.80$; 6.19 ± 0.25 vs 6.09 ± 0.29 for RRC-NI and RRC-NLA, respectively). However, the BK dose response E_{max} value for COT arteries in the presence of NLA was significantly greater in the CCC-NLA ($P = 0.01$; 88.49 ± 7.92), RCC-NLA ($P < 0.01$; 87.16 ± 6.52), and RRC-NLA ($P < 0.01$, 85.09 ± 5.44) arteries compared with CCC-NI (52.67 ± 7.92), RCC-NI (53.69 ± 7.29), and RRC-NI (59.17 ± 5.44) arteries.

Caruncular and Cotyledonary Arterial Responses to Bradykinin

The CRCs comparing the vasodilatory effects of BK in the CAR and COT placental arteries had a significant treatment by dose interaction in the RCC-CAR vs RCC-COT ($P < 0.0001$) (Figure 4.8B) and RRC-CAR vs RRC-COT ($P < 0.03$) (Figure 4.8C) arteries while a treatment effect ($P < 0.0001$) was observed when CCC-CAR arteries when compared with CCC-

COT arteries (Figure 4.8A). The CAR placental arteries were more sensitive to BK induced vasodilation while, in the COT placental arteries, relaxation was initially inhibited and arteries reached a stable plateau until the 3×10^{-8} M dose of BK; after which sentivity was enhanced at the subsequent BK doses. The COT arteries responded similarly to the CAR arteries at the highest dose (3×10^{-6} M) of BK.

Maternal nutrition influenced the pD_2 values in the COT placental arteries. The CCC-COT ($P < 0.02$; 6.54 ± 0.42), RCC-COT ($P < 0.0001$; 6.19 ± 0.19), and RRC-COT ($P < 0.001$; 6.71 ± 0.31) had significantly higher pD_2 values compared with CCC-CAR (8.48 ± 0.36), RCC-CAR (8.81 ± 0.19), and RRC-CAR (9.05 ± 0.31) arteries. However, the E_{max} values for the CAR and COT placental arteries were similar ($P = 0.43$; 38.33 ± 11.66 vs 52.67 ± 12.77 for CCC-CAR and CCC-COT, respectively; $P = 0.49$; 41.06 ± 11.78 vs 53.69 ± 13.17 for RCC-CAR and RCC-COT, respectively; $P = 0.26$; 42.53 ± 9.78 vs 59.17 ± 9.78 for RRC-CAR and RRC-COT, respectively) across treatments.

BK2R mRNA Expression

Maternal dietary treatment did not significantly influence the mRNA expression of BK2R in the CAR ($P = 0.48$; 1.38, 1.33, 1.51 ± 0.11 arbitrary units for CCC, RCC, and RRC, respectively) and the COT ($P = 0.73$; 1.21, 1.36, 1.21 ± 0.16 arbitrary units for CCC, RCC and RRC, respectively) placental arteries.

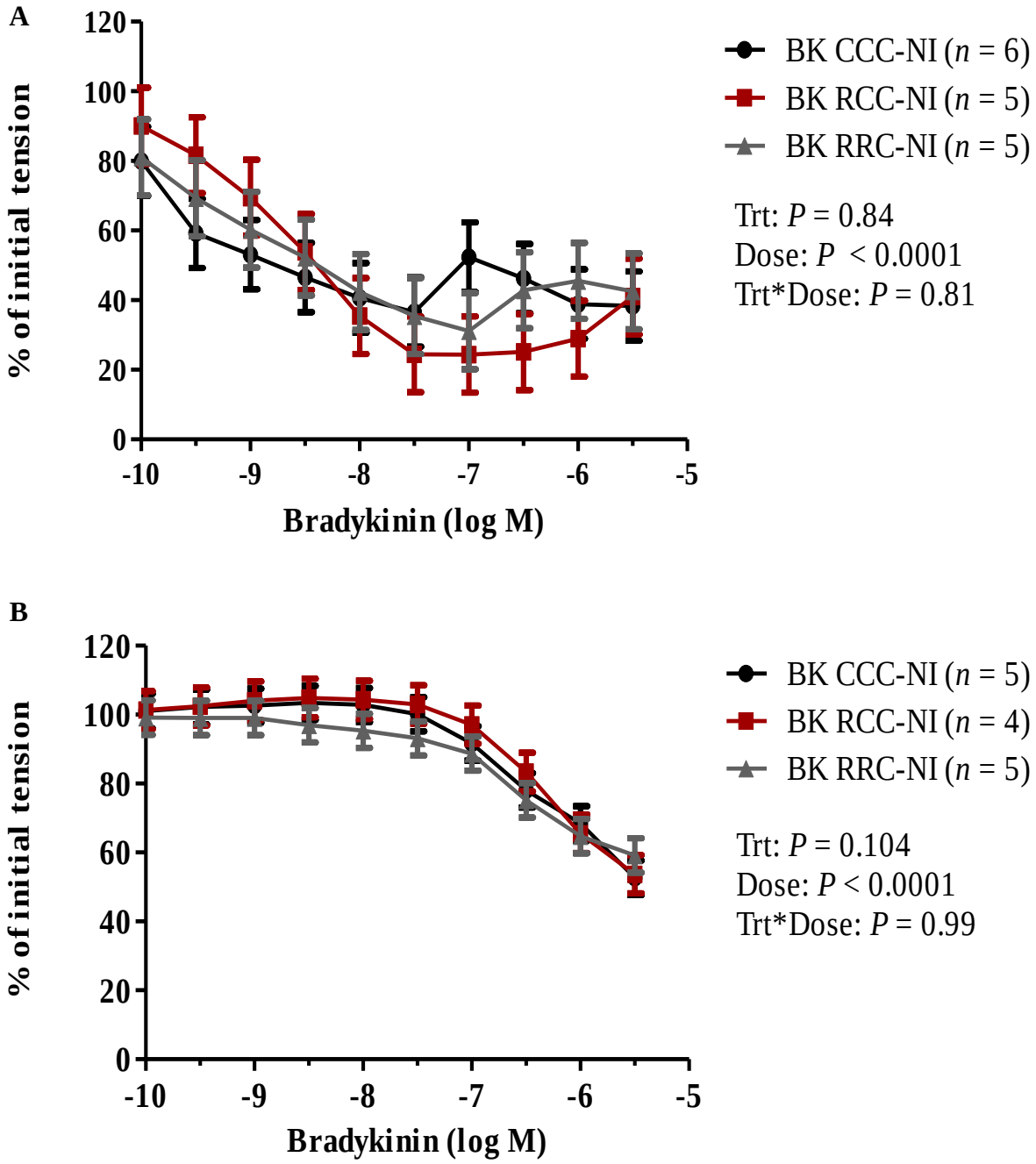

Figure 4.1. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in (**A**) caruncular (**CAR**) arteries and (**B**) cotyledonary (**COT**) arteries. Cows received 100% (**CCC**) NRC requirements from d 30 to d 254, 60% (**RCC**) NRC requirements from d 30 to d 85 with realimentation to 100 % NRC requirements on d 85 until d 254, and 60% (**RRC**) NRC requirements from d 30 to d 140 with realimentation to 100% NRC requirements on d 140 until d 254 of gestation. Figure compares BK concentration response curve obtained in the absence of any inhibitor (**NI**) (CCC-NI vs RCC-NI vs RRC-NI).

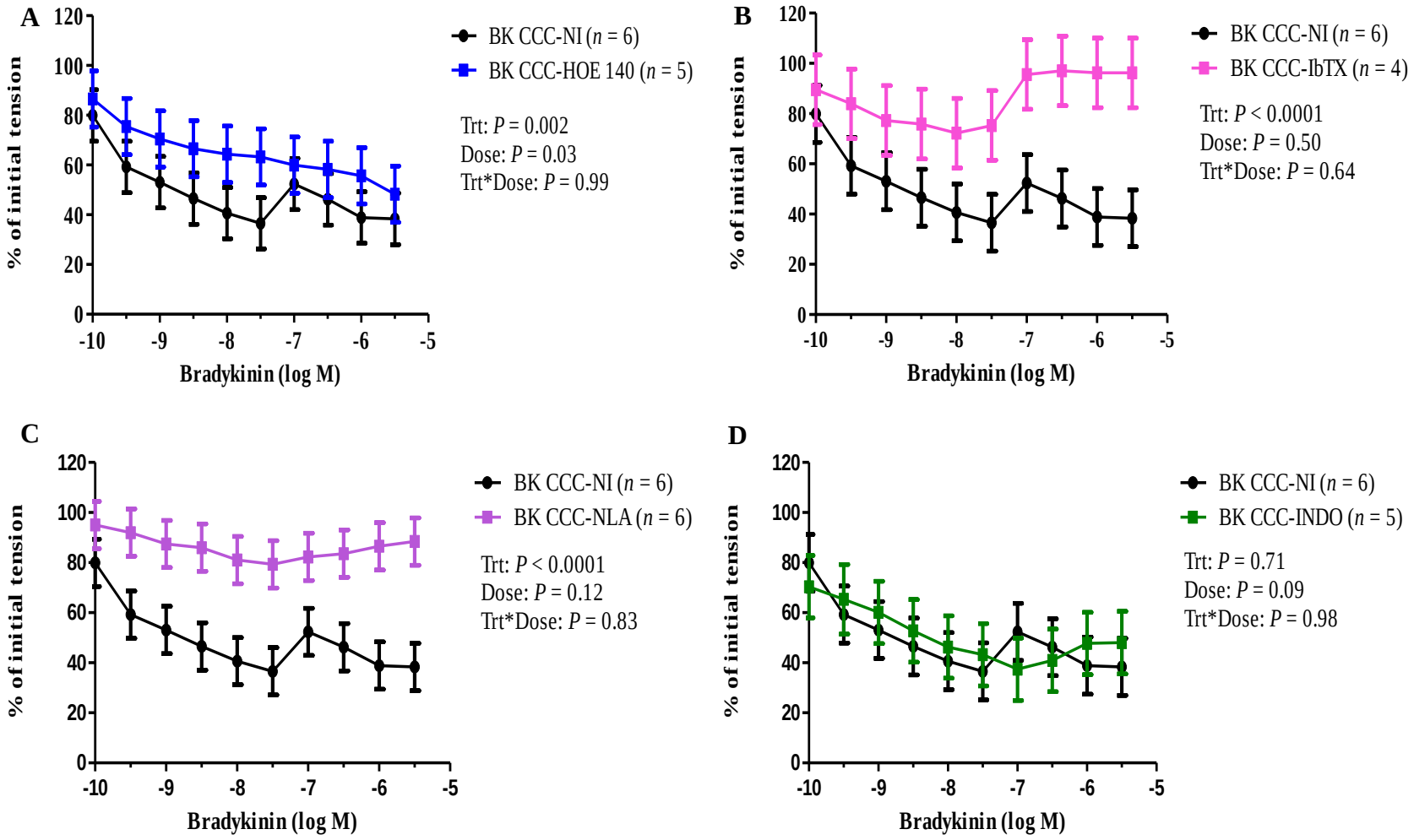

Figure 4.2. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in caruncular (**CAR**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**D**) after incubation with indomethacin (**INDO**). Cows received 100% (**CCC**) NRC requirements from d 30 to d 254 of gestation.

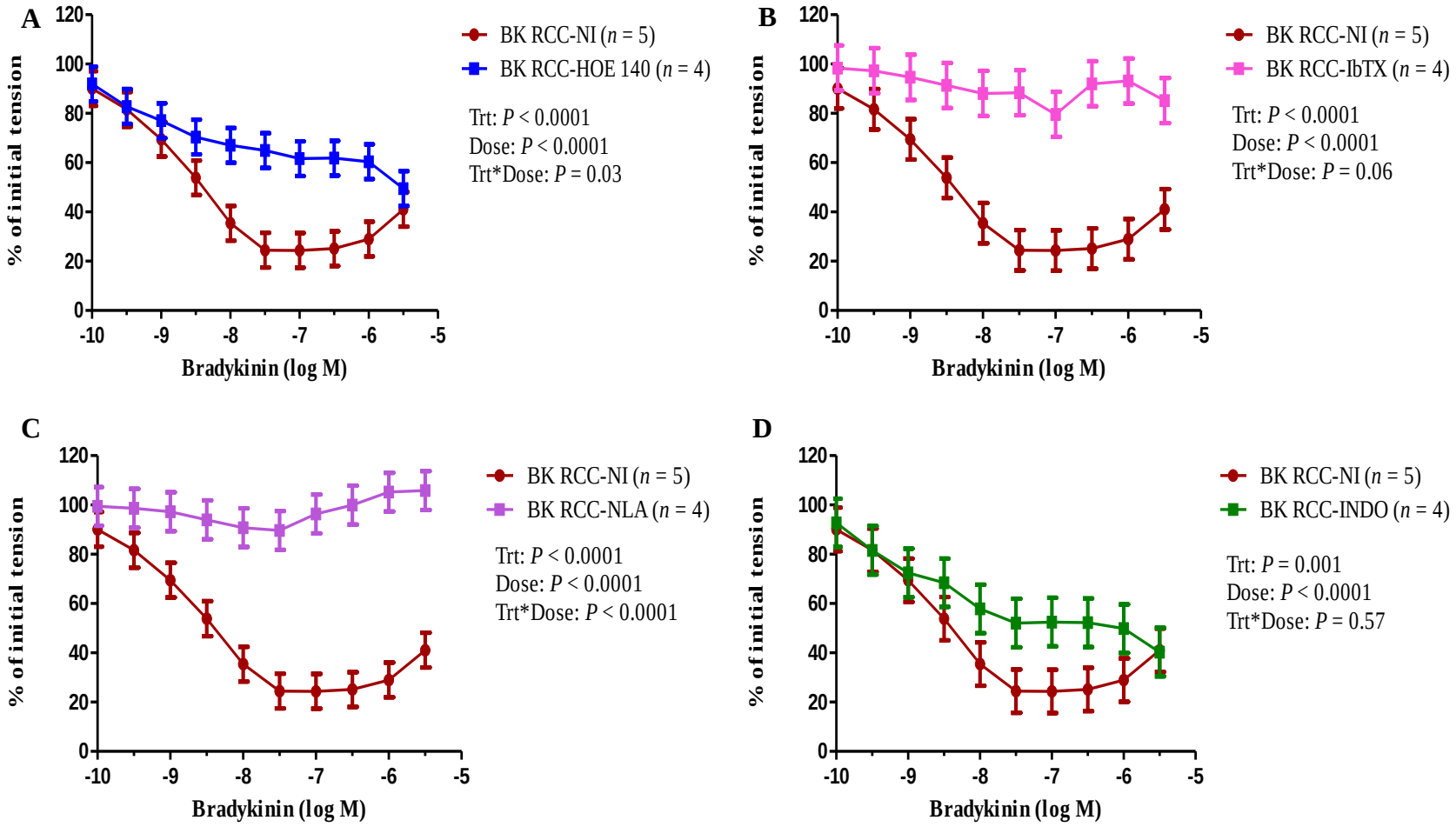

Figure 4.3. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in caruncular (**CAR**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**D**) after incubation with indomethacin (**INDO**). Cows received 60% (**RCC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 254 of gestation.

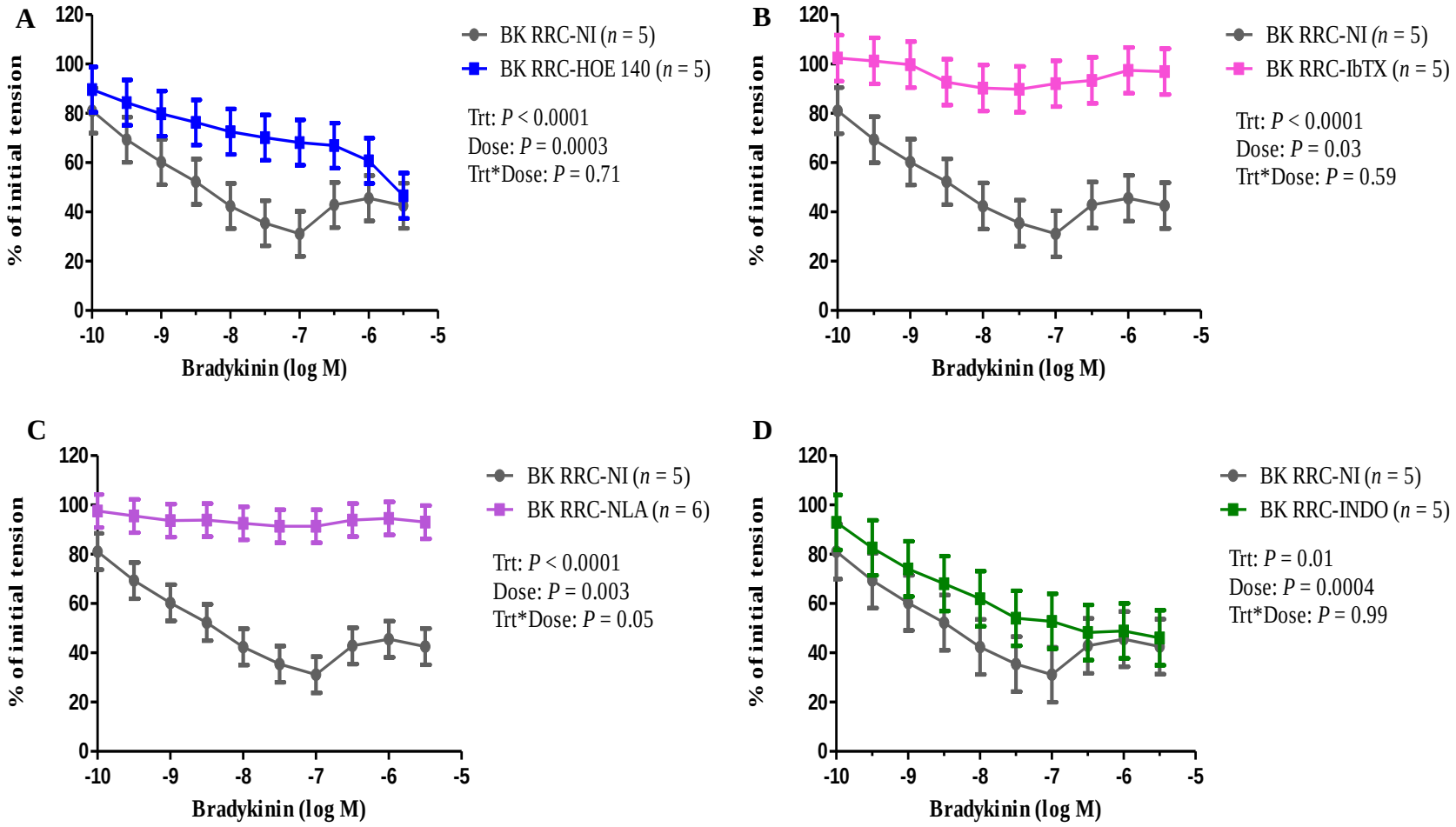

Figure 4.4. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in caruncular (**CAR**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**), and (**D**) after incubation with indomethacin (**INDO**). Cows received 60% (**RRC**) NRC requirements from d 30 to d 140 with realimentation to 100% NRC requirements on d 140 until d 254 of gestation.

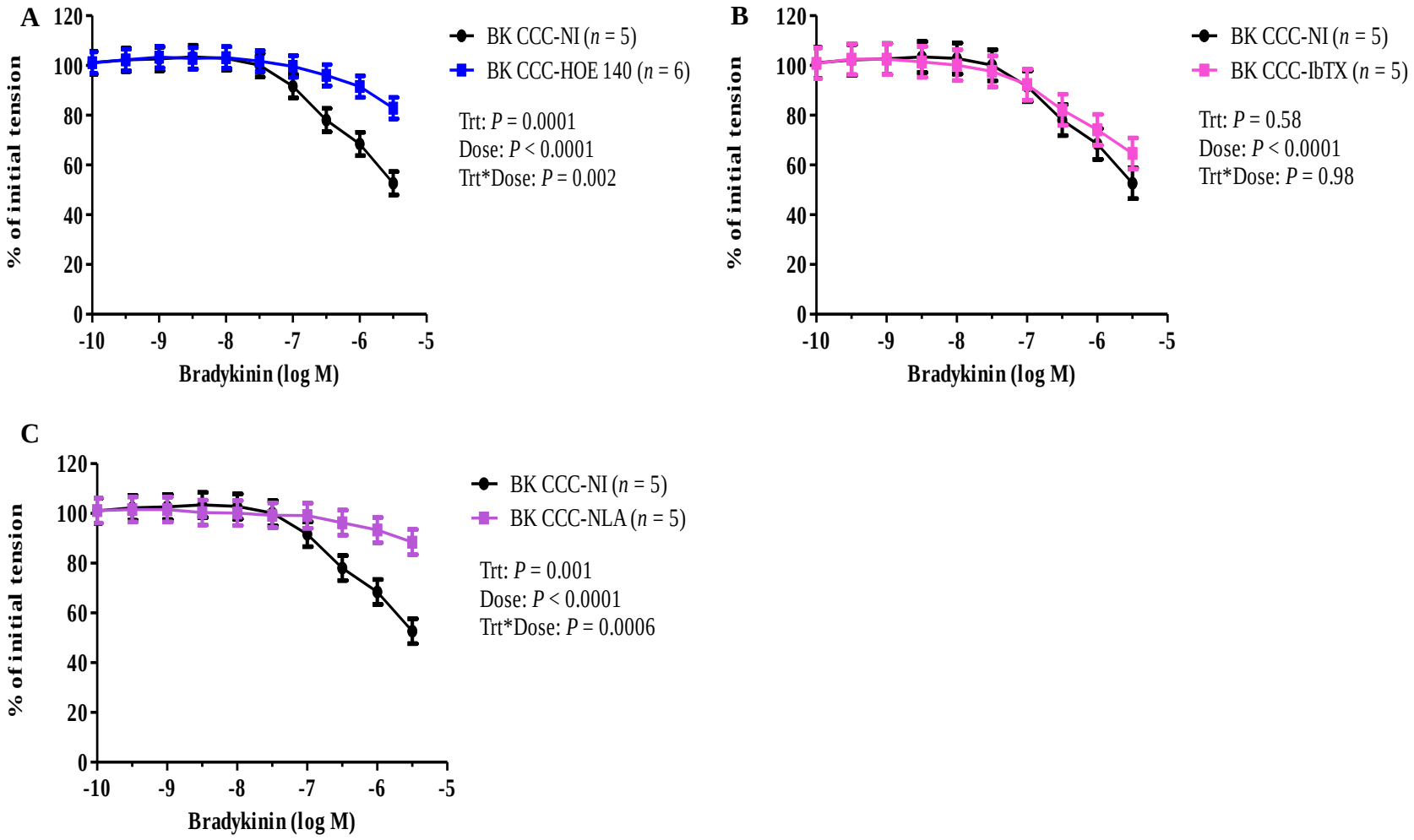

Figure 4.5. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), and (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**). Cows received 100% (**CCC**) NRC requirements from d 30 to d 254 of gestation.

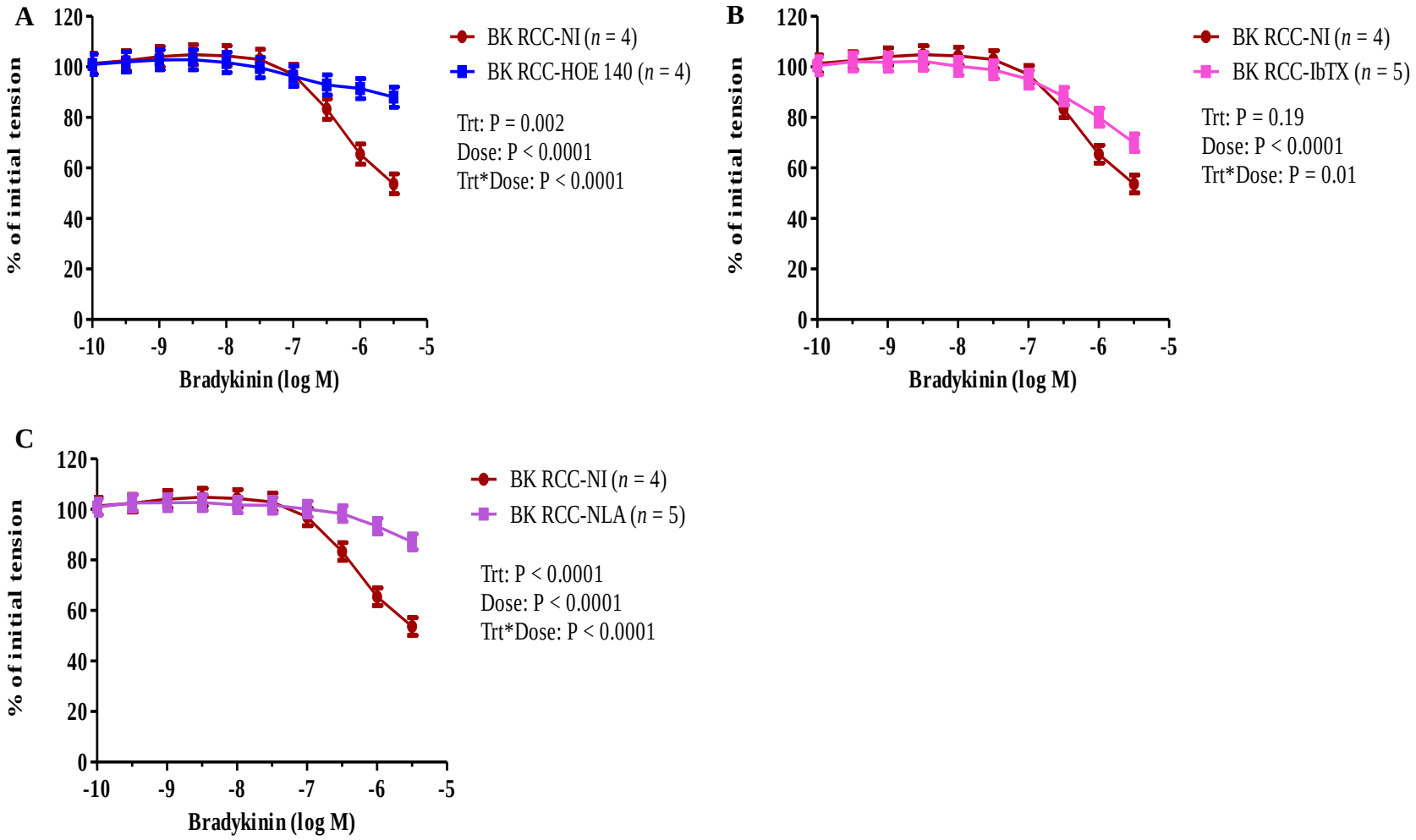

Figure 4.6. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), and (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**). Cows received 60% (**RCC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 254 of gestation.

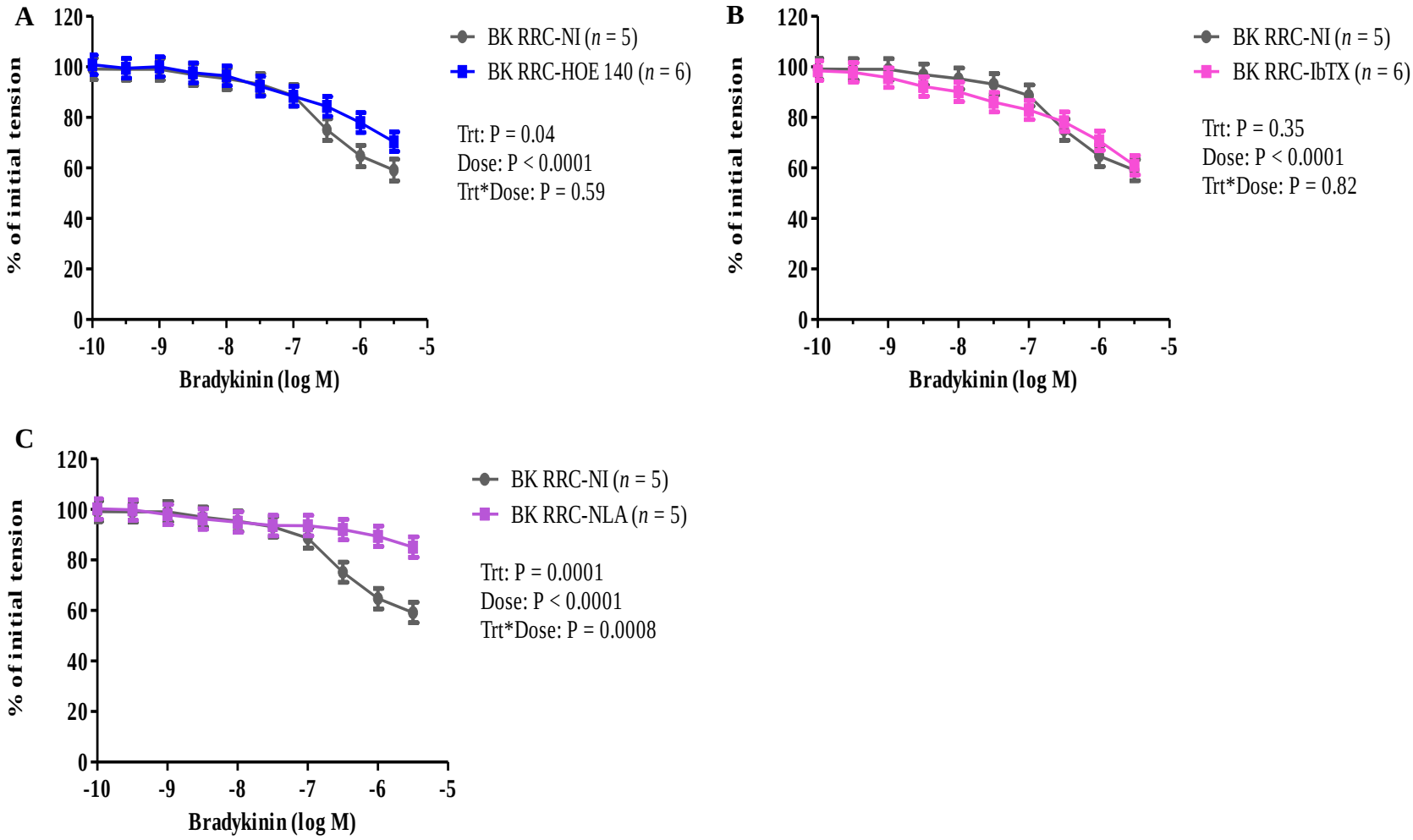

Figure 4.7. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in cotyledonary (**COT**) arteries with no inhibitor (**NI**) compared with arteries (**A**) after incubation with icatibant acetate (**HOE 140**), (**B**) after incubation with iberiotoxin (**IbTX**), and (**C**) after incubation with *N*(nitro)-L-arginine (**NLA**). Cows received 60% (**RRC**) NRC requirements from d 30 to d 140 with realimentation to 100% NRC requirements on d 140 until d 254 of gestation.

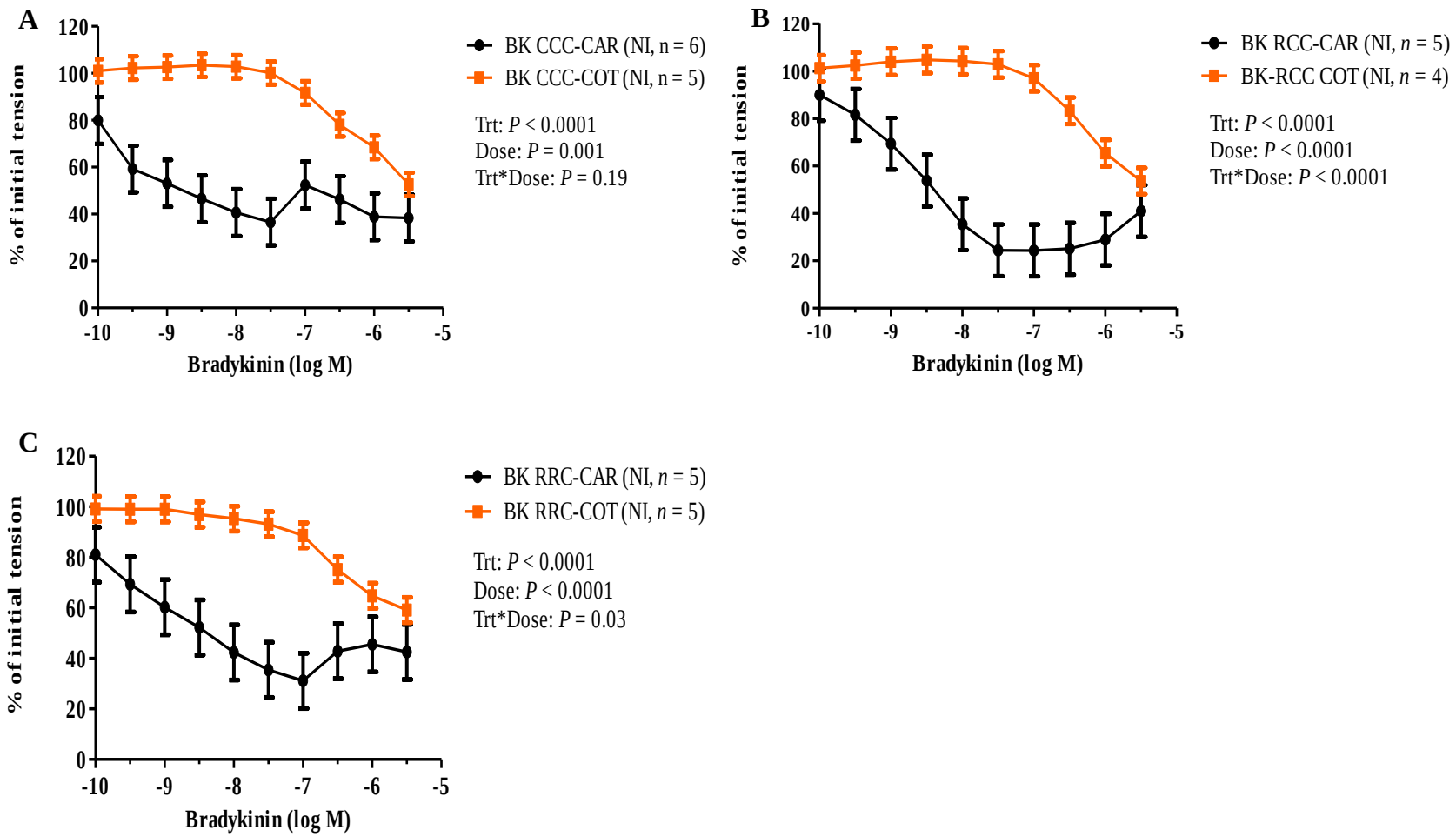

Figure 4.8. The impacts of maternal nutrition and bradykinin (**BK**) dose on the BK concentration response curve in the caruncular (**CAR**) and cotyledonary (**COT**) arteries without an inhibitor (**NI**) in cows receiving (**A**) 100% (**CCC**) NRC requirements from d 30 to d 254 of gestation, (**B**) 60% (**RCC**) NRC requirements from d 30 to d 85 with realimentation to 100% NRC requirements on d 85 until d 254 of gestation, and (**C**) 60% (**RRC**) NRC requirements from d 30 to d 140 with realimentation to 100% NRC requirements on d 140 until d 254 of gestation.

Discussion

As described previously, maternal nutrient restriction during early (d 30 to d 85; Chapter 2) and mid-gestation (d 30 to d 140; Chapter 3) increased the sensitivity of the CAR and the COT placental arteries to vasorelaxation in response to the endothelium dependent vasodilator, BK. These findings represent an important underlying mechanism by which dietary treatment may impact placental vasoactivity altering nutrient availability to the developing calf. This possibly depicts the presence of a compensatory mechanism employed by the placenta to ensure optimum nutrient delivery to the fetus induced as a result of maternal nutrient restriction during early and mid-gestation. However, during late gestation maternal diet had no effect on placental vasoactivity, and nutrient restriction followed by realimentation did not produce any changes in the CAR and the COT placental arteries which responded similarly across treatments. It is of interest to note that near term, the placental arteries have completely adapted after the two durations of nutrient restriction followed by realimentation and are responding similarly to the control arteries. Moreover, the mechanisms employed for mediating relaxation in the CAR and COT placental arteries are different. The CAR placental arteries had delayed relaxation at lower doses of BK in the presence of HOE 140; however, at the highest dose the arteries responded similarly indicative of an adaptive response or perhaps regulation of vasodilation via the BK1R inconjuction with the BK2R. In the COT placental arteries, incubation with HOE 140 yielded a higher E_{max} value in the CCC and the RCC cows suggesting an elevation in the role of the BK2R. While, relaxation was delayed in the RRC arteries at lower doses, no differences in E_{max} values were observed indicative of an alternate mechanism working in conjunction with the BK2R for mediating vasodilation in RRC cows.

Furthermore, BK_{Ca} channels are involved in mediating relaxation in the CAR arteries; while, no effect was observed in the COT arteries upon incubation with IbTX. In the current study, the COT placental arteries relaxed despite blocking the BK_{Ca} channels. Since, the EDHF(s) are representative of a diverse group of chemical compounds comprising of L-citrulline, K^+ ion, H_2O_2, anandamide, and epoxyeicosatrienoic acids (Tanaka et al., 2004), it is quite possible that the observed relaxations are likely due to an EDHF that is not inhibited by IbTX. As gestation advances, the placenta is required to keep pace with the ever increasing fetal demands for growth and development. Thus, relaxation produced in the COT placental arteries might be an adaptive response to ensure optimum nutrient delivery to the fetus. Therefore, further investigation of EDHF(s) is required which might provide a potential mechanism responsible for mediating BK induced relaxation in COT placental arteries in the presence of IbTX.

In the presence of NLA, relaxation was inhibited in both, the CAR and the COT arteries pointing out the pivotal role of NO in mediating vasodilation in the placental arteries. The E_{max} value of the CAR and the COT placental arteries incubated with NLA were significantly greater implying that the primary vasodilator is likely NO released from the endothelial cells in response to BK.

In the CAR placental arteries, in presence of INDO, although relaxation was inhibited in the RCC and the RRC arteries at a few doses of BK; the pD_2 and E_{max} were values were similar across treatments indicating that INDO does not prevent the BK induced vasorelaxation in the CAR placental arteries, and perhaps BK does not elicit vasorelaxation through the PGI_2 pathway in the CAR placental arteries.

The mRNA expression of the BK2R analyzed in the present study did not differ across the three treatment groups in both the CAR and the COT placental arteries, implying that the receptor gene expression is not altered by nutritional status. It remains unknown how BK1R may change due to nutritional adaptation which might be a stress induced response to overcome nutrient restriction in the CAR placental arteries. These findings further emphasize the importance of vasoreactivity assays rather than relying on mRNA expression data as the sole determinant.

Lastly, it is of interest to note that maternal nutrient restriction during early and mid-gestation followed by realimentation significantly enhances BK induced relaxation in the CAR placental arteries at lower doses. Overall, the COT placental arteries had a greater pD_2 value suggesting that relaxation is delayed when compared with the CAR placental arteries. Similar to our findings at d 85 and d 140 of gestation, the CAR vascular bed is observed to be more sensitive to vasodilation at lower doses of BK than the COT vascular bed near term. This increased sensitivity could allow for an efficient nutrient delivery system and help support the exponential growth of the fetus during the last half of gestation (Metcalfe, 1988; Ferrell, 1989; Reynolds and Redmer, 1995).

Taken together, the results of the present study do not support our hypothesis that global maternal nutrient restriction followed by realimentation in pregnant beef cows alters sensitivity of the maternal and the fetal arteries to BK induced relaxation. It appears that placental arteries have completely adapted after the two durations of nutrient restriction followed by realimentation and are responding similarly to the control arateries. Despite this, there may be different durations of restriction which may program the placenta to compensate vasodilation by different mechanisms. Although, relaxation is not influenced by the dietary treatment across

treatments it is mediated at least partly by the BK2R and its pathways mediating BK induced vasodilation in the placental arteries. Future investigations are required in the area of bovine maternal and fetal placental development and how potential therapeutics may impact compromised pregnancies in a cow.

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CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Conclusion

There are currently very limited studies investigating the effects of global maternal nutrient restriction and realimentation on placental vascular function in the bovine. The current study is novel as it not only focuses on the effects of maternal nutrient restriction but also realimentation across gestation. While, early and mid-gestation are crucial times for placental establishment and growth, late gestation is marked by maximal fetal growth. Since, the placenta plays a pivotal role in developmental programming; any insults to the maternal system have the capacity of altering placental function (Barker, 1997). Inadequate placental function could lead to intrauterine growth restriction (IUGR) (Chandler et al., 1985; Barker et al., 1990; Barker et al., 1993; Wallace et al., 2002; Carr et al., 2012; Lemley et al., 2012) resulting in life-long alterations in the postnatal life (Godfrey and Barker, 2000). Therefore, an understanding of factors that impact the placental vascular development and function with relation to utero-placental blood flow would influence the nutritional and physiological status of the offspring both, *in utero* and in postnatal life.

Our findings suggest that a period of nutrient restriction during early and mid-gestation does not prove to be detrimental as it alters the vascular function of the placental arteries in the cow. The restricted animals are able to overcome the loss of nutrients via placental compensation while realimentation returns placental arteries vasoactivity similar to control cows in response to BK.

There are currently no good therapeutics for IUGR, and this is mainly due to a lack of knowledge on the mechanisms of IUGR. The results from this study provided novel information

which would help in the development of potential therapeutics aimed at enhancing placental efficiency which would potentially be beneficial in overcoming compromised pregnancies (Reynolds et al., 2006, 2010).

Overall, these studies indicate that global maternal nutrient restriction during early and mid-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 in the arteries are likely mediated through different pathways.

Future Directions

The data in this study contributes to the small number of studies regarding global maternal nutrient restriction and realimentation during gestation on placental vascular function in the cow. However, 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 due to restriction of a specific component in the diet. Since, variable levels of dietary protein have been shown to have an effect on various components of the placenta (Perry et al., 1999; Sullivan et al., 2009); therefore, future studies investigating the role of different levels of dietary protein on placental vascular reactivity across gestation are important.

The current study also provides novel information on how BK 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 exactly detect how big of a role each of the endothelium-dependent pathways has in placental vasorelaxation and whether these pathways are inter-related. Since in the present study the effects of bradykinin 1

receptor (BK1R) were not analyzed, additional vasoactivity studies investigating the role of the BK1R and perhaps a combination of the BK1R and the bradykinin 2 receptor inhibitors which would help in the better understanding of the specific role played by each receptor type across gestation.

The fetal placental arteries relaxed in the presence of inhibitor to large conductance Ca^{2+} activated K^{+} channels. Thus, further investigation of endothelial-derived hyperpolarizing is required to provide a potential BK induced vasodilatory mechanism in the fetal placental arteries. The effects of prostacyclins should be tested in the fetal placental arteries since; there was limited space in the wire myograph in the current study.

Additionally, analysis of mRNA and protein expression of the BK1R, endothelial nitric oxide synthase and its receptor, soluble guanylate cyclase, could help answer some questions from the present study. Finally, similar studies to better understand the mechanisms of BK induced vasodilation in the placental arteries are needed if future therapeutics for compromised pregnancies will target the placental vascular beds.

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