

C-Type Natriuretic Peptide Forms in Pregnancy: Maternal Plasma Profiles during Ovine Gestation Correlate with Placental and Fetal Maturation

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Circulating concentrations of C-type natriuretic peptide (CNP) and a related amino terminal fragment (NTproCNP) were measured at weekly intervals from preconception to 3 wk postpartum in ewes with twins ($n = 8$) and nonpregnant ewes ($n = 8$). In contrast to low and stable values in nonpregnant ewes (CNP, 0.75 ± 0.08 ; NTproCNP, 22 ± 2 pmol/liter), CNP forms increased abruptly at 40–50 d of gestation and rose to peak values (CNP, 31 ± 5 , NTproCNP, 270 ± 16 pmol/liter) at about d 120. Approximately 7 d prepartum, the concentration of both CNP forms fell precipitously to preconception values immediately postpartum. In separate studies, circulating maternal CNP forms were positively related to fetal number at d 120. Consistent with a major contribution from the placenta to circulating levels, the concentrations of CNP forms were elevated in the placentome (cotyledon: CNP, 18 ± 4 , NTproCNP, 52 ± 10 pmol/g; caruncle: CNP, 13 ± 3 , NTproCNP, 31 ± 6 pmol/g) and much higher than those of intercaruncular uterine tissue (CNP, 0.19 ± 0.05 , NTproCNP, 0.98 ± 0.2 pmol/g) in late-gestation ewes ($P < 0.001$, $n = 4$). These distinctive patterns of maternal plasma CNP forms, positive relation with fetal number, and greatly elevated protein concentrations in the placentome demonstrate the hormone's strong relation to placental and fetal maturation. The findings provide a firm basis for future studies of the functional role of CNP in fetal-maternal welfare. (*Endocrinology* 150: 4777–4783, 2009)

C-type natriuretic peptide (CNP), a member of the natriuretic peptide family of structurally related peptides, is a paracrine/autocrine growth factor with important roles in cardiovascular homeostasis and skeletal development. CNP and its receptor, natriuretic peptide receptor-B, have also been identified in uterine and placental tissues and in both male and female gonads (reviewed in Ref. 1). Moreover, in mice, uterine CNP mRNA expression is stimulated by estradiol (2), and CNP concentration in ovarian tissues fluctuates with the estrous cycle (2). These findings suggest that CNP may be of special importance in female reproductive biology.

Recent studies by Prickett *et al.* (3) showed that maternal circulating concentrations of CNP are remarkably

increased (some 50-fold) in late gestation ewes when compared with nonpregnant control sheep. Plasma concentrations of the (presumably) biologically inactive form of CNP, amino-terminal proCNP (4) (NTproCNP), were also elevated at least 10-fold. A major contribution by uteroplacental tissues to these high systemic concentrations in late gestation was suggested by high arteriovenous CNP gradients across the gravid uterus (3) and significant correlation between maternal plasma NTproCNP concentration and placental weight (3). However, the respective contributions from placental and uterine tissues have not been determined.

Postulating that in sheep the time course of maternal plasma concentration of CNP forms throughout preg-

nancy may provide insights to placental and fetal maturation, we have now undertaken a longitudinal study, from preconception to parturition, of maternal plasma CNP forms in healthy ewes and compared the findings to normal nonpregnant ewes studied under identical conditions. The hypothesis that CNP secretion has a positive relationship with fetal number was also tested by measuring maternal circulating CNP and NTproCNP concentration in late gestation ewes with single, twin, and triplet pregnancies. Finally, to clarify the likely source of CNP in the maternal circulation, we examined the peptide content of placental and intercaruncular tissues from late gestation ewes.

Materials and Methods

Ethics

All procedures involving animals were approved by the Lincoln University Animal Ethics Committee.

Part one: longitudinal survey

Fifty-three mixed-age Coopworth, or Coopworth × South-Hampshire cross ewes (*Ovis aries*) were randomly selected from the Lincoln University Research Farm for this study. Animals were grazed outdoors at the Lincoln University Research Farm (Canterbury, New Zealand), with supplementary feed supplied during winter. Synchronization of the estrous cycle was achieved using intravaginal progesterone-releasing devices (Eazi-breed type G controlled internal drug releases; Pfizer New Zealand Ltd., Auckland) in accordance with a published protocol (5). On the day of mating, ewes were randomly allocated into two groups and run with two crayon-harnessed intact rams (pregnant group, $n = 32$) or with a crayon-harnessed vasectomized ram (control group $n = 21$). Five weeks later, the rams were removed and pregnant and nonpregnant animals were run together for the remainder of the study. Confirmation of pregnancy was determined by ultrasonography in early gestation and subsequent birth of a lamb. Because conception dates were estimates, results are expressed in relation to actual parturition date. Blood samples (10 ml) were collected weekly beginning 1 wk before conception until 3 wk postpartum for measurement of CNP, NTproCNP, estradiol, and progesterone. Non-fasting live weights were recorded at intervals of 2 wk.

Part two: effect of number of fetuses

Mixed-age natural-mated Suffolk, Perendale, or Corriedale ewes from the Lincoln University Research Farm were examined by transabdominal ultrasonography at approximately d 94 of pregnancy. Ewes were marked according to the number of fetuses detected by the scanning procedure. At approximately d 120 of pregnancy, ewes with a single, twin, or triplet pregnancy (20 of each category) were selected at random and a single blood sample was collected from each of the 60 animals.

Part three: CNP forms in utero-placental tissue

Four mixed-age Coopworth ewes from the Lincoln University Research Farm carrying a single lamb were killed by captive bolt

gun on approximately d 143 of pregnancy. Before slaughter, a maternal blood sample was collected from each of the four ewes. A fetal blood sample was collected immediately after slaughter. The uterus was excised and four randomly selected placentomes were removed from each ewe as well as four samples of intercaruncular uterine tissue. To determine the individual contribution of maternal and fetal tissues to placental CNP concentration, the maternal (caruncle) and fetal (cotyledon) aspects of the placentome were separated at the time of tissue collection. This was achieved by gently pulling on the two sides of the placentome, yielding a clean separation of the two tissues. After dissection, the placental and uterine tissues were immediately snap frozen in liquid nitrogen and stored at -80 C until required.

Blood samples

Blood collection was by jugular venipuncture into 10-ml evacuated blood tubes containing EDTA as anticoagulant (Vacutainer, Franklin Lakes, NJ) that were immediately placed on ice. Tubes were centrifuged at $800 \times g$ for 10 min at 4 C, and then plasma was transferred into polystyrene tubes and stored at -20 C until required. The time from collection until centrifugation did not exceed 2 h.

Extraction and characterization of peptides from placental and uterine tissue

CNP and NTproCNP were extracted from samples of frozen uterine and placental tissue (~ 1 g) as previously described (6). To characterize placental molecular forms, representative tissue samples were extracted using Sep-Pak C 18 cartridges (Waters Corp., Milford, MA) as described previously (3). Extracts were resuspended in 20% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid before size exclusion (G2000; ToyoSoda, Tokyo, Japan) HPLC.

Hormone assays

In all cases, the entire set of samples from an individual animal was processed in duplicate in a single assay to minimize any impact of interassay variation. Because initial studies showed evidence of nonparallelism at high concentrations of placental NTproCNP, care was taken to ensure that only readouts from validated portions of the standard curve were used.

RIA for CNP

CNP was assayed as previously described (3, 7), with minor changes as follows: 50 μ l standard or sample extract was preincubated with 50 μ l of a commercial primary rabbit antiserum raised against proCNP (82-103) (Phoenix Pharmaceuticals Inc., Belmont, CA; catalog no. RAB-014-03) and diluted to 1:3000, to which 50 μ l of tracer (1500 cpm) were added after a 22- to 24-h incubation period. Within- and between-assay coefficients of variation were 6.6 and 8.6%, respectively, at 1.1 pmol/liter. The detection limit and EC_{50} for this assay were 0.4 and 6.1 pmol/liter, respectively.

RIA for NTproCNP

NTproCNP was measured as previously described (3, 4) with the following alterations: 100 μ l standard/sample extract was preincubated with 50 μ l primary rabbit antiserum raised against NTproCNP (1–15) and diluted to 1:6000 (J39), to which 50 μ l of tracer (1500 cpm) were added after a 22- to 24-h incubation

period. Within- and between-assay coefficients of variation were 7.5 and 7.9%, respectively, at 24 pmol/liter. The detection limit and EC_{50} for this assay were 1.3 and 58 pmol/liter, respectively.

Progesterone

Plasma progesterone concentration was measured in triplicate 50- μ l aliquots by ELISA as previously described (8). Within- and between-assay coefficients of variation were 6.1 and 14.9%, respectively.

Estradiol

Plasma estradiol concentration was measured by RIA (E2-Estradiol-2; Sorin Biomedica, Saluggia, Italy). Within- and between-assay coefficients of variation were 9.7 and 20%, respectively, at 41 pmol/liter. The detection limit and EC_{50} for this assay were 4.5 and 130 pmol/liter, respectively.

Statistical analyses

Statistical analyses were performed using Systat version 7.0 (SPSS Inc., Chicago IL) or Genstat version 10 (VSN International Ltd., Hemel Hempstead, UK). Repeated-measures ANOVA was used to assess changes in maternal CNP and NTproCNP with time as the independent variable. One-way ANOVA was used to determine a difference in hormone concentration with number of fetuses. Bonferroni *post hoc* analyses were performed for both the longitudinal and number of fetuses studies. Restricted maximum likelihood and a method of statistical differentials were used to detect differences in hormone concentration in utero-placental tissues. A paired *t* test was used to determine differences between maternal and fetal circulating hormone concentrations at d 143. Data were log transformed where appropriate, and a $P < 0.05$ was considered statistically significant.

Results

An excess number of ewes was included in the longitudinal study to allow for animals failing to become pregnant, death or illness of mother and/or neonates, and differing number of fetuses. Because single fetuses were underrepresented, their number being insufficient to allow statistical evaluation, the study groups were confined to healthy twin-bearing ewes with normal pregnancy and delivery and healthy nonpregnant control ewes. From each of these two groups, eight animals were chosen at random for completion of hormone profiles (Figs. 1 and 2).

Live weight and birth weights of lambs

All animals used in the longitudinal study had weight gain profiles and birth weights consistent with normal pregnancy for Coopworth sheep on this farm.

Circulating CNP and NTproCNP concentrations throughout pregnancy

Plasma concentrations of CNP and NTproCNP increased significantly during pregnancy in ewes ($P < 0.01$; Fig. 1). One week before conception, plasma CNP concentration was

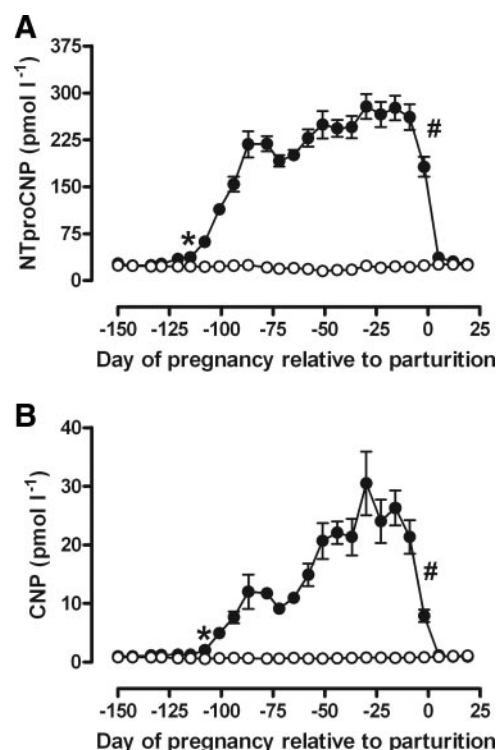


FIG. 1. Mean (\pm SEM) plasma NTproCNP (A) and CNP (B) concentration in twin-bearing pregnant (●) and nonpregnant (○) ewes ($n = 8$). Day of pregnancy refers to pregnant animals. *, Significant difference from prepregnancy baseline; #, significant difference between the final 2 wk of gestation. Parturition occurred at d 0.

0.98 ± 0.06 pmol/liter and plasma NTproCNP concentration was 27.2 ± 1.07 pmol/liter. In pregnant ewes, circulating concentrations of CNP and NTproCNP became significantly elevated above prepregnancy values by 115 and 110 d before parturition, respectively (ovine gestation length is ~ 145 d) (CNP, $P < 0.001$; NTproCNP, $P < 0.05$). Peak plasma concentration of CNP (31 ± 5 pmol/liter) and NTproCNP (270 ± 16 pmol/liter) was observed at around 30 d before parturition, followed by a significant decline in the concentration of both peptide forms (7 d before parturition; CNP, 36% of peak, $P < 0.001$; NTproCNP, 71% of peak, $P < 0.01$). Circulating CNP and NTproCNP concentrations returned to prepregnancy values immediately after parturition ($P > 0.1$).

In nonpregnant ewes, the plasma concentration of CNP and NTproCNP (mean \pm SEM) was 0.75 ± 0.08 and 22 ± 2 pmol/liter, respectively. There was a small but significant fall in plasma concentration of CNP ($P < 0.05$) and NTproCNP ($P < 0.001$) during the study period (Fig. 1).

Circulating estradiol and progesterone concentrations in pregnant ewes

Mean plasma estradiol concentration declined steadily during the first trimester (Fig. 2A). Thereafter the values fluctuated but there was a sustained elevation during the final 3 wk of pregnancy before falling sharply at parturition. In-

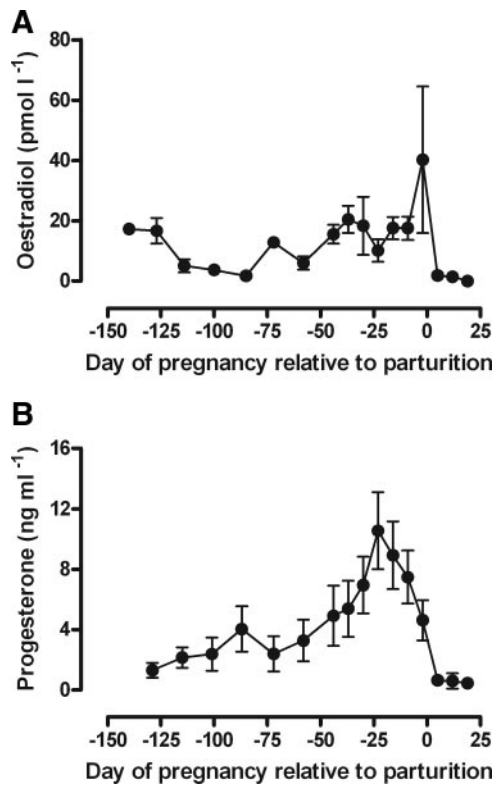


FIG. 2. Mean (\pm SEM) plasma estradiol (A) and progesterone (B) in twin-bearing pregnant ewes ($n = 8$) throughout the course of gestation. Parturition occurred at d 0.

crease in mean plasma progesterone concentration commenced soon after midgestation, peaking approximately 23 d before parturition, after which the mean concentration declined progressively (Fig. 2B). After parturition both estradiol and progesterone were barely detectable in the maternal venous plasma.

Effect of fetal number

The number of fetuses had a significant positive effect on the concentration of circulating CNP forms, being higher in triplet pregnancies (CNP, $P < 0.001$; NTproCNP, $P < 0.001$) (Fig. 3).

Identity and concentration of CNP forms in utero-placental tissues

When subjected to size exclusion HPLC/RIA, a major peak of immunoreactive CNP eluted in a position consistent with CNP-53 (Fig. 4A). A smaller peak (fractions 31–33) consistent with CNP-22 was also identified. As shown in Fig. 4B, a major peak of immunoreactive proCNP 1-15 eluted in a position consistent with the 5-kDa (or closely similar) aminoterminal fragment (proCNP 1-50). A smaller (unidentified) and later eluting peak was also present. The early eluting shoulder (fractions 22–25) of immunoreactive material identified in both Fig. 4, A and B, is consistent with the presence of proCNP (1–103).

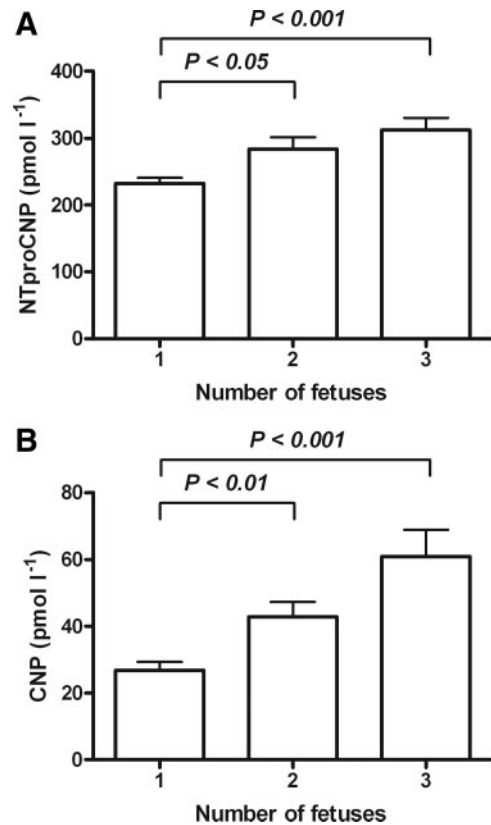


FIG. 3. Mean (\pm SEM) plasma concentration of NTproCNP (A) and CNP (B) at around d 120 of gestation in ewes carrying one ($n = 23$), two ($n = 20$), or three ($n = 17$) fetuses.

CNP and NTproCNP concentrations in the placentome were significantly higher than in uninvolved uterine tissue (CNP, $P < 0.001$; NTproCNP, $P < 0.001$) (Fig. 5). Within the placentome, the concentration of both CNP forms was significantly higher in the fetal cotyledon compared with maternal caruncle (CNP, $P < 0.05$; NTproCNP, $P < 0.001$) (Fig. 5). Plasma CNP and NTproCNP concentrations were higher in the maternal compared with fetal circulation at around d 143 of pregnancy (CNP, $P = 0.01$, NTproCNP, $P = 0.01$) (Fig. 6).

Discussion

This report constitutes the first longitudinal study of maternal circulating CNP forms throughout pregnancy in any species. In confirming previous findings of greatly raised concentrations in late gestation in the ewe (3), the present results extend our knowledge of CNP's participation in ovine pregnancy by showing an abrupt increase coinciding with placentation followed by a progressive rise until near term and a marked decline in maternal concentrations during the final week of pregnancy. In addition, we show a direct relationship between maternal concentration of CNP forms and fetal number and identify the placenta as the major contributor to

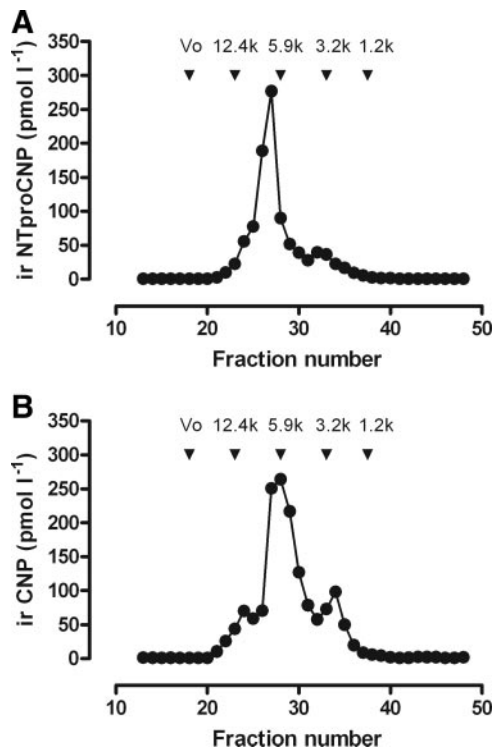


FIG. 4. Immunoreactive (ir) NTproCNP (A) and CNP (B) size exclusion HPLC profiles of ovine placental extract from d 143 of gestation. Column void volume (V_0) and elution positions of molecular markers are shown by arrows.

the high gradient across the gravid uterus. These observations strongly support a role for CNP in placental and fetal maturation and serve as a basis for future *in vivo* studies of CNP's functional role.

In ewes bearing twins, venous plasma NTproCNP concentration increased abruptly toward the end of the first trimester in strong contrast to the much lower and unchanging concentration of NTproCNP in nonpregnant adult control animals. The initial rate of increase in plasma NTproCNP concentration was greater than that of CNP, but increases in both forms slowed about 90–60 d before parturition (at a gestational age of 55–85 d). Of interest, similar patterns of change are observed in plasma nitric oxide (9) and may be associated with reduced estrogen production during the same period (10). After midgestation, the rate of increase in maternal CNP concentration exceeded that of NTproCNP. Whether these differential changes in CNP forms relate to reduced activity of degradative pathways, including natriuretic peptide receptor-C (which is down-regulated in uterine arteries in late gestation) (11) or neprilysin or possibly associated with the phase of rapid fetal growth is unclear. Although CNP synthesis is high in the fetus, any contribution of the fetus itself to maternal levels is unlikely because the fetal lamb plasma concentration of CNP (3.3 ± 0.5 pmol/liter, Fig. 6) is much lower than maternal levels and appears to be regulated independently (3).

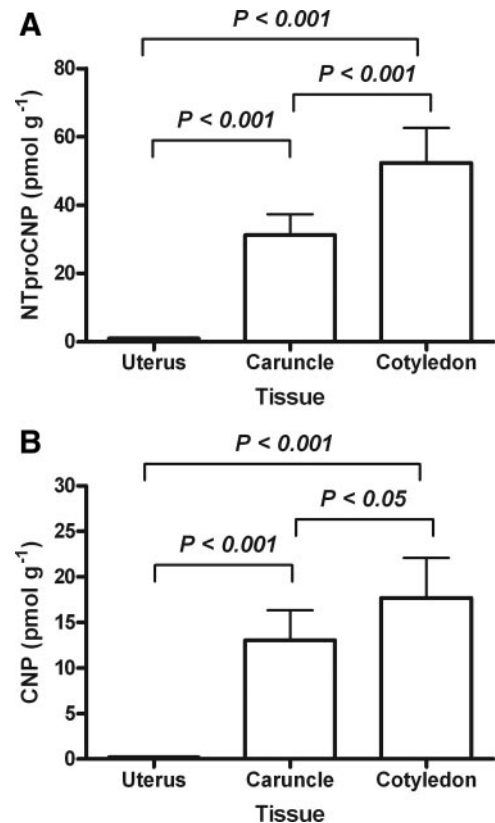


FIG. 5. Mean (\pm SEM) concentration of NTproCNP (A) and CNP (B) in the uterus, maternal placenta (caruncle), and fetal placenta (cotyledon) of single-bearing ewes at around d 143 of gestation ($n = 4$).

Our study was not designed to determine the mechanisms underlying the dramatic changes we observe in maternal concentrations nor their functional consequences. Nonetheless, the time sequence, close correlation of plasma concentration of CNP forms with fetal number, and the greatly elevated CNP concentrations within the placenta all point to the placenta as a major source. Of note, the initial increase in maternal NTproCNP concentration corresponds to the phase of maximum placental growth and invasion of the maternal stroma (12). In mice, we have shown abundant CNP expression closely associated with the maternal blood vessels of the placenta (13), possibly initiated by increases in shear stress (14). This localization suggests a role for the hormone in angiogenesis (15, 16) and vasodilation (17). Indeed, later in gestation it seems likely that both the local (auto/paracrine) actions within placenta and uterus (1, 2) as well as endocrine contributions from high circulating CNP concentrations (18) all contribute to the massive increase in utero-placental blood flow associated with the pregnant state (11, 19). In this context, increased estrogen production is known to be important in maintaining vasodilation (20). The effects of estrogens are mediated in part by nitric oxide, but the changes in this pathway are considered to be insufficient to account for the increases in uterine blood

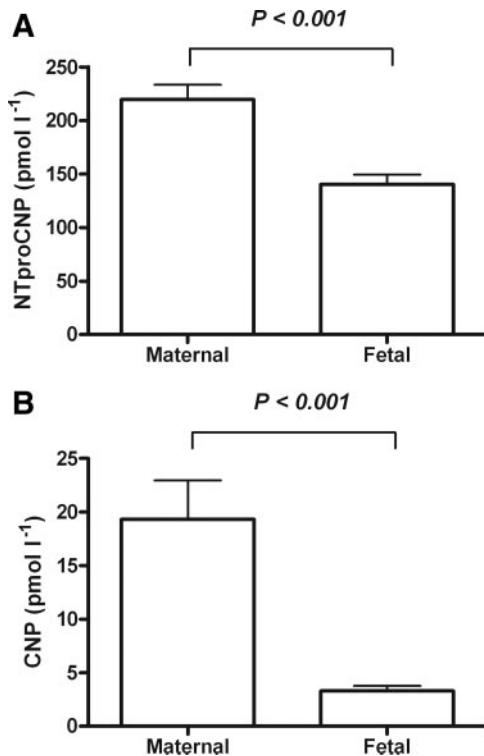


FIG. 6. Mean (\pm SEM) plasma concentration of NTproCNP (A) and CNP (B) in single-bearing ewes and their fetuses at around d 143 of pregnancy ($n = 4$).

flow observed in late gestation (20). Estradiol increases CNP gene expression in murine uterus (2) and, as recently reported by us, also stimulates plasma CNP forms in adult (nonpregnant) ewes (21). Together these findings raise the possibility that some of estradiol's actions on uterine blood flow may be mediated by CNP. Whereas this may be true after midgestation, it is notable that the initial increases in NTproCNP occur during a period of declining plasma estradiol concentration, making it unlikely that estradiol is the main driver of CNP synthesis in pregnancy.

Our finding of significantly higher CNP protein concentrations in placental compared with uterine tissue in late gestation is consistent with other reports in mice (22). Importantly, analyses using HPLC-RIA confirmed that the molecular forms in placental tissues at term are closely similar to those previously reported in the ovine maternal circulation (3). The fact that in sheep, unlike humans, maternal plasma CNP appears to reflect placental synthesis of the hormone offers a novel approach to the study of placental CNP regulation *in vivo*. Furthermore, surgical techniques isolating uterine from uteroplacental vein collections should allow studies of the factors regulating CNP production in these individual tissues (and its functional role) in the living animal. Of note, cotyledonary concentrations of both CNP forms exceed those in the caruncle, suggesting that CNP, like ovine placental lactogen (23), may be synthesized by the fetal binucleate cell (syncytiotrophoblasts). These cells migrate across

the placenta to release the products into the maternal circulation. Such a fetal maternal gradient within the placenta would also be consistent with recent results from our laboratory (24), showing that the fetal concentrations of CNP forms in matched umbilical and venous plasma are similar in late gestation.

A striking observation in the present study is the precipitous decline in concentrations of both CNP forms during the final week of pregnancy. The time sequence of these changes coincides with the period of rapid hypothalamus-pituitary-adrenal activation in the fetus (25). The rise in fetal plasma cortisol concentration in lambs commences around 15 d before delivery (25) and is associated with progressive induction of placental 17 α -hydroxylase and fall in maternal plasma progesterone as observed in our study. Conceivably the upsurge in fetal cortisol secretion in the final 3–5 d (25) may be sufficient at the placental tissue level to down-regulate CNP expression. We have shown previously that glucocorticoids can rapidly inhibit CNP synthesis *in vivo* (6, 26). Whether similar inhibition can occur at the much lower concentrations of glucocorticoids attained near term awaits further study. In any event, the current findings suggest that inhibiting CNP once the fetus is mature may be an important step in preparing for parturition, *i.e.* by restoring myometrial contractility (1, 27) and reducing vasodilation (17) and potential blood loss associated with placental separation.

The current findings in pregnant ewes, together with evidence of reciprocal changes in fetus (in which CNP falls) and ewe (in which CNP rises) during nutritional stress (3), strongly support the view that CNP is carefully regulated in pregnancy and that change in maternal plasma CNP concentration, at least in sheep, may reflect local uteroplacental secretion and fetal distress. On this hypothesis, higher maternal circulating CNP concentration with increasing fetal number in ewes at d 120 gestation may be seen as an adaptive change to caloric restriction. This view is reinforced by our finding that the fetal plasma CNP concentration is lower in a twin when compared with that in a single fetus (3). In late-gestation ewes carrying twin and triplet fetuses, nutritional requirements are 120 and 140% above maintenance, respectively (28), which if not met, will result in caloric restriction. Similar mechanisms may be the basis for higher maternal concentrations of NTproCNP, and lower fetal values, in women presenting with preeclampsia when compared with normal pregnant women at term (29). Clearly these hypotheses now require experimental support, possibly using models of placental insufficiency.

In conclusion, the distinctive pattern of maternal concentrations of CNP forms (abrupt rise at the end of the first trimester and sudden prepartum fall), positive relation

with fetal number, and greatly elevated concentrations in the placentome demonstrate the hormones' strong relation to placental and fetal maturation. Together the findings provide a basis for elucidating the functional role of CNP in fetal-maternal welfare.

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