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The trophoblast binucleate cell is the source of maternal circulating C-type natriuretic peptide during ovine pregnancy

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ABSTRACT

Maternal plasma concentrations of C-type natriuretic peptide (CNP) and a co-secreted bioinactive amino-terminal fragment (NTproCNP) are elevated during ovine pregnancy. Although the uteroplacental unit has been implicated as a likely source of CNP, the relative contributions of specific uterine and placental tissues, and identity of the cellular site/s of production remain unknown. Therefore, we measured CNP and NTproCNP in intercaruncular uterine tissue and maternal (caruncle) and fetal (cotyledon) placental tissues throughout gestation. Concentrations of CNP forms in placental tissues greatly exceeded those in intercaruncular uterine tissue throughout pregnancy ($P < 0.05$). Mean caruncular concentrations (CNP 32 ± 4, NTproCNP 56 ± 6 pmol g^{-1}) peaked at day 60 whereas in the cotyledon there was a progressive increase in CNP forms to peak values (CNP 66 ± 6, NTproCNP 134 ± 9 pmol g^{-1}) at day 100–135 followed by a sharp decline just prior to term (day 143). At term CNP gene expression was 6-fold greater in placental tissue compared with intercaruncular uterine tissue. Changes in maternal plasma concentration of CNP forms closely followed those in cotyledonary tissue whereas fetal plasma levels fell progressively throughout gestation. Immunohistochemistry revealed staining in binucleate cells (BNC) and around placental blood vessels.

CNP's localization to the BNC suggests a novel endocrine role during pregnancy, in addition to its paracrine actions within the placental vasculature. The function of CNP in maternal circulation remains to be determined, but as proposed for other BNC products, may involve manipulation of maternal physiology and placental function to favour fetal growth.

1. Introduction

C-type natriuretic peptide (CNP) is a growth and vasoactive factor that circulates in healthy adults at barely detectable concentrations of <1 pmol l^{-1} [1]. However, in ovine and cervine pregnancy, maternal plasma concentrations of CNP and of the presumed bioinactive amino-terminal fragment of proCNP (NTproCNP) are substantially elevated [2,3]. Although the role of CNP in pregnancy is unclear, studies in human pregnancy show differential responses of placental and uterine CNP gene expression in settings of IUGR or preeclampsia [4]. Further, the abrupt increase in maternal plasma CNP concentration as fetal levels fall during nutrient restriction in sheep [5] supports the view that CNP is carefully regulated in pregnancy. Activity of the CNP signalling pathway is selectively augmented in the uterine artery during ovine pregnancy [6] – raising the possibility that CNP contributes to the greatly increased uteroplacental blood flow associated with normal pregnancy. Taken together these findings suggest that as well as maintaining nutrient supply during normal gestation, the hormone may also serve to improve nutrient supply in the setting of fetal distress [5].

In sheep, concentrations of CNP increase at around gestational day 40 to levels exceeding 20 pmol l^{-1} before rapidly declining during the final week of gestation [2]. The temporal pattern of CNP forms in maternal circulation [2] and high venoarterial concentration gradient across the gravid uterus [5] strongly implicate the uteroplacental unit as an important source of maternal CNP during pregnancy. However the relative contributions from uterine and placental tissues are unknown, as is the identity of the cell type/s within the placenta responsible for CNP production.

A unique feature of the ruminant placenta is the presence of large, granulated binucleate cells (BNC) of fetal placental origin which appear at the time of implantation and persist for the remainder of pregnancy. BNC are formed from uninuclear

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trophoblast cells that differentiate into the binucleate phenotype and are released from the trophoblast basement membrane. In ovine pregnancy, the mature BNC migrate through the trophoblast tight junction and fuse with uterine epithelial cells forming a feto-maternal hybrid syncytium [7]. The cytoplasmic contents of the BNC fuse into the fetomaternal syncytium and the contents of BNC granules are released by exocytosis at the basal plasmalemma of the original uterine epithelial cell, from where they diffuse into the maternal circulation.

Recent results from our laboratory demonstrated that CNP and NTproCNP protein concentration in the fetal component of the placenta (cotyledon) exceeded that in the maternal aspect (caruncle) in near-term ewes, despite a higher peptide concentration in maternal versus fetal plasma [2]. This finding, taken with the similarity between the pattern of CNP production during pregnancy and abundance of BNC in the ovine placenta [7], suggests that CNP may be a product of these unique cells of pregnancy. Therefore, the aim of the current study was to characterise the pattern of CNP expression in uterine and placental tissue at early, mid and late pregnancy and to address the hypothesis that maternal circulating CNP is produced by fetal binucleate cells [reviewed in 8,9].

2. Materials and methods

2.1. Ethics

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

2.2. Blood samples

Blood samples were collected by jugular venipuncture (ewes) or cardiac puncture (fetuses) into 10 ml evacuated blood tubes containing K_3EDTA (Vacutainer, Franklin Lakes, NJ, USA). Samples were immediately placed on ice, centrifuged at 4 °C, and stored at −20 °C until required. The time from collection until centrifugation did not exceed two hours.

2.3. Tissue samples

Uterine and placental tissues were collected as described previously [2]. Briefly, mixed-age Coopworth ewes from the Lincoln University Research Farm carrying single ($n = 9$) or twin ($n = 11$) lambs were killed by captive bolt gun on day 30, 60, 100, 135 and 143 of pregnancy ($n = 4$ at each time-point, term = day 145). In the case of twins, samples of plasma and placental tissue were collected from each fetus and the hormone concentration averaged to give a single value for that animal. Prior to slaughter, a maternal blood sample was collected from each ewe. Fetal blood samples were collected immediately after slaughter at all time-points except day 30, when the fetal blood volume was too low to enable measurement of CNP forms. The uterus was excised and four randomly-selected placentomes were removed from each ewe as well as four samples of intercaruncular uterine tissue. At the time of tissue collection, maternal (caruncle) and fetal (cotyledon) aspects of the placentome were separated by gently pulling on the two sides of the placentome, yielding a clean separation of the two tissues. At day 135, samples of maternal skeletal muscle (gracilis), intercaruncular uterine tissue and placental tissue were also collected for measurement of CNP gene expression. After dissection, all tissues were immediately frozen by immersion in liquid nitrogen and stored at −80 °C. Blood and uterine caruncular and intercaruncular tissue samples were also collected from non-pregnant Coopworth ewes at similar times (corresponding to days 30 and 135 in the pregnant group, $n = 4$ at each time-point) and stored in an identical manner as for the pregnant ewes.

2.4. CNP gene expression

Total RNA was isolated using TRIzol (Invitrogen). Tissue samples (~300 mg) were homogenised by grinding in a Retsch MM301 tissue mill at 30 Hz for 10 min in 800 μl TRIzol. First-strand cDNA was synthesized from 1 μg total RNA with Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR analyses were performed with a Rotor-Gene RG-3000 real-time PCR machine (Corbett Research, Sydney, Australia). The CNP transcript was detected and quantified with the following set of primers: 5′-CAGAAGAAGGGCGACAAGAC-3′ (sense) and 5′-CCCTTGGACAAACCCTTCTT-3′ (antisense) – amplified product size 179 bp. PCRs were performed in a total volume of 20 μl containing 1 μl cDNA, 0.4 mM primers, 0.3 μl AccuPrimeTaq DNA polymerase (Invitrogen), 1× PCR buffer I, 0.2 mM deoxynucleotide triphosphates, 4 μl 5×Q solution (Qiagen) and 1 μl (1:5000 dilution) Sybr Green 1 (Roche). Following a hot start at 96 °C for 2 min, each sample underwent 45 cycles of annealing at 60 °C for 35 s, extension for 30 s at 72 °C, and denaturation at 90 °C for 30 s. Samples were assayed in duplicate and gene expression levels quantified against a standard curve and expressed as picograms of message per microgram of total RNA.

2.5. Extraction of peptides from placental and uterine tissue

CNP and NTproCNP were extracted from samples of frozen uterine and placental tissue (approximately 1 g) as previously described [2].

2.6. Hormone assays

2.6.1. RIA for CNP

CNP was assayed as previously described [5,10], with minor changes as follows: 50 μl standard or sample extract was preincubated with 50 μl of a commercial primary rabbit antiserum raised against proCNP (82–103) (Phoenix Pharmaceuticals Inc., Belbont, CA, USA. Catalogue #RAB–014–03) and diluted to 1:3000, to which 50 μl of tracer (1500 cpm) was added after a 22–24 h incubation period. Within- and between-assay coefficients of variation were 6.6% and 8.6% respectively at 1.1 pmol l^{-1}. The detection limit and EC50 for this assay was 0.4 and 6.1 pmol l^{-1} respectively.

2.6.2. RIA for NTproCNP

NTproCNP was measured as previously described [1,5] with the following alterations: 100 μl standard or sample extract was preincubated with 50 μl primary rabbit antiserum raised against NTproCNP (1–15) and diluted to 1:6000 (J39), to which 50 μl of tracer (1500 cpm) was added after a 22–24 h incubation period. Within- and between-assay coefficients of variation were 7.5% and 7.9% respectively at 24 pmol l^{-1}. The detection limit and EC50 for this assay was 1.3 and 58 pmol l^{-1} respectively.

2.7. Cross-reactivity

Cross-reactivity with either ovine placental lactogen or pregnancy associated glycoprotein was <0.0009% for both the CNP and NTproCNP assays.

2.8. Immunohistochemistry

In order to investigate site/s of CNP localisation within the placentome, whole placentomes were collected at approximately day 124 of pregnancy from twin-bearing Coopworth ewes, frozen in liquid nitrogen and stored at −80 °C before being processed for immunohistochemistry. Placentomes were embedded in optimal cutting temperature compound and sectioned at 6 μm. Endogenous peroxidise activity was blocked by incubation in 1% peroxide solution for 20 min at room temperature. Slides were washed three times in phosphate buffered saline solution (PBS) containing 1% normal goat serum and incubated with primary antibody (CNP 1:250; NTproCNP 1:500) or PBS (negative control) overnight at 4 °C. The slides were then subjected to successive overnight incubations at 4 °C- first with biotinylated secondary antibody (1:1000) followed by a second incubation with an ExtraAvidin-HRP complex (1:1000) (Sigma–Aldrich). A PBS washing step was performed between each incubation. The slides were then washed in HRP reaction buffer prior to staining using HRP substrate solution (30 min incubation followed by dehydration steps using a series of alcohols and xylene) in preparation for viewing by microscopy.

To determine whether CNP is a product of the trophoblast BNC, additional studies were performed in Cambridge using araldite resin-embedded sections to locate CNP, NTproCNP and pregnancy associated glycoprotein (PAG). PAG is produced only by the fetal trophoblast BNC and therefore served as a positive control for identification of these cells. Two Welsh Mountain ewes were mated naturally and killed with an overdose of sodium pentothal at 80 days of pregnancy, in accordance with the UK Animals (Scientific Procedures) Act, 1986. After laparotomy the uterus was cut out, the fetus removed and the uterine and umbilical arteries cannulated in turn and perfused with 1% glutaraldehyde plus 4% formaldehyde in 0.1 M phosphate buffer and finally embedded in epoxy (araldite) resin by standard procedures [11]. For immunocytochemistry, 1 μm resin sections were picked up on aminopropyltriethoxysilane-coated coverslip pieces and dried at 60 °C for 30 min. Resin was removed from the epoxy resin sections by flotation on sodium ethoxide (15 g NaOH in 15 ml absolute ethanol) for 15 min. The sections were washed with 100% alcohol, and then sequentially floated on 50% alcohol, PBS, PBS plus 1% bovine serum albumin (the blocking solution and diluent for all antibodies), and finally on antibody solution (polyclonal CNP 1:50; NTproCNP 1:50; PAG M4 1:1000 [11]; monoclonal SBU3 1:1000 [11]) overnight at 4 °C.

After mouse monoclonal antibody incubation and a PBS wash the sections were floated on rabbit anti-mouse serum (Dako Ltd., Ely, Cambridgeshire, UK) diluted 1:1000 for 30 min, washed with PBS and then joined by the polyclonal incubations on goat anti-rabbit 5 nm gold colloid solution (Stratech Scientific, Soham, Cambridgeshire, UK; diluted 1:40) for 40 min. All sections were washed with PBS