N-terminal pro-C-type natriuretic peptide, but not C-type natriuretic peptide, is greatly elevated in the fetal circulation

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ABSTRACT

We have identified recently a new peptide, NT-proCNP(1–50) (N-terminal pro-C-type natriuretic peptide), in the circulation of humans and sheep. A previous report of an elevated fetal–maternal gradient in immunoreactive CNP raised the possibility that processing and metabolism of proCNP may differ in maternal and fetal tissues. We therefore collected matching peripheral maternal and umbilical cord plasma samples at delivery from women with normotensive and pre-eclamptic pregnancies to investigate the presence and concentrations of CNP and NT-proCNP using HPLC and RIA. Plasma concentrations of NT-proCNP in normotensive umbilical cord plasma were 10-fold higher than maternal venous levels (246 ± 17 compared with 24.3 ± 1.8 pmol/l; \( P < 0.001 \)) and much higher than corresponding levels of CNP (3.6 ± 0.4 compared with 1.8 ± 0.3 pmol/l in the fetal and maternal plasma respectively; \( P < 0.001 \)). Although there was no significant difference between normotensive and pre-eclamptic plasma CNP concentrations in either maternal or umbilical cord blood, NT-proCNP showed a significant statistical interaction (\( F = 5.8, P = 0.025 \)) between the source (maternal or fetal) and gestational group (normotensive or pre-eclamptic). Maternal NT-proCNP levels were raised in the pre-eclampsia group, whereas the converse was observed in umbilical cord blood. In conclusion, the greatly elevated ratio of NT-proCNP/CNP in fetal compared with maternal plasma suggests that synthesis, as well as clearance, of CNP (but not NT-proCNP clearance) are markedly increased in fetal tissues.

INTRODUCTION

The natriuretic peptides, ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide) and CNP (C-type natriuretic peptide), constitute a family of structurally related peptides which share a ring structure containing 17 amino acids. CNP was first identified in porcine brain tissue [1] and has subsequently been identified in a variety tissues, including the pituitary [2], pineal gland [3], vascular endothelium [4], VSMCs (vascular smooth muscle cells) [5], kidney [6], testis [7], ovary and uterus [8]. CNP has been shown to have a number of biological actions. It is, for example, a vasodilator and an inhibitor of growth in VSMCs [9]. Elevated levels in blood occur in severe sepsis and renal disease [10], raising the possibility that endothelial disorders are associated with enhanced release of the hormone, but the precise role of CNP in homeostasis remains to be clarified.

CNP is synthesized as a 103 amino acid pro-hormone (proCNP), which is cleaved between residues 50 and 51 or 81 and 82 to release the biologically active C-terminal peptides CNP-53 and CNP-22 [11]. These peptides are

Key words: cord blood, C-type natriuretic peptide (CNP), N-terminal pro-C-type natriuretic peptide (NT-proCNP), placental tissue, pre-eclampsia.

Abbreviations: CNP, C-type natriuretic peptide; NPR, natriuretic clearance receptor; NT-proCNP, N-terminal pro-CNP; RP, reverse phase; SE, size exclusion; VSMC, vascular smooth muscle cell.

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rapidly cleared from the circulation (half-life = 2.6 min) [12], due to degradation by the natriuretic clearance receptor (NPR-3) and the enzyme neprilysin (neutral endopeptidase). We have shown previously [13] that an N-terminal peptide fragment of proCNP (NT-proCNP) is also released into the circulation. This peptide had a molecular mass consistent with it being NT-proCNP(1–50).

Recently, immunoreactive CNP was identified in the fetal circulation at concentrations significantly greater than those found in the maternal circulation of normal women [14]. Given these findings and that NT-proCNP is unlikely to be metabolized by NPR-3 or neprilysin, we predicted that NT-proCNP levels would be markedly elevated in umbilical cord plasma. Accordingly, we have measured levels of NT-proCNP and CNP in fetal and maternal plasma as well as umbilical and placental tissues. Because endothelial dysfunction is commonly associated with pre-eclamptic states, we also compared CNP and NT-proCNP levels in plasma drawn from pregnant women without and with pre-eclampsia. We hypothesized that the higher circulating levels of NT-proCNP, which are more accurately measured than CNP, may better reflect CNP production in the vasculature. Thus NT-proCNP measurement may show significant differences not revealed by CNP measurement.

**MATERIALS AND METHODS**

**Subjects**

We studied 23 Caucasian women, mean age 31 years (range 26–35 years) at the time of delivery, in the Department of Obstetrics and Gynecology, Helsinki University Hospital, Finland. Ten were normotensive and 13 were pre-eclamptic, as defined by blood pressure greater than 140/90 mmHg with proteinuria according to a dipstick test or 0.3 g/24 h after 20 weeks of gestation (Table 1). None of the women studied had pregnancies complicated by intrauterine growth retardation. Maternal venous blood was collected into tubes containing EDTA immediately after delivery, centrifuged and the plasma stored at −80 °C. Blood from the umbilical cord was gently emptied into tubes containing EDTA, centrifuged and the plasma stored at −80 °C. Plasma samples were air freighted as a single batch on dry ice to the Cardioendocrine Research Laboratory in Christchurch, New Zealand for analysis. The study was approved by the Ethics Committee of the Helsinki University Women’s Hospital and all participants gave written informed consent.

**Extraction of peptides from plasma and tissue**

Maternal or cord plasma samples (1–2 ml) were extracted using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, U.S.A.) as described previously [15]. Placental or umbilical cord tissue (1–2 g) was boiled, homogenized and extracted on Sep-Pak C18 cartridges, as previously described for ovine pituitary tissue [16], and dried under an air stream. Extracts were re-suspended in either assay buffer for RIA, or 0.1 % TFA (trifluoroacetic acid) or 20 % (v/v) acetonitrile in 0.1 % TFA prior to RP-(reverse-phase; G18) or SE- (size-exclusion; G3000; Toyo Soda, Tokyo, Japan) HPLC respectively.

**RIA for NT-proCNP**

The NT-proCNP assay developed by us was performed as described previously [13], except that a more sensitive primary rabbit antiserum (J39) raised against human NT-proCNP(1–15) was used, and peptide standards were made from synthetic human proCNP(1–19) taking into account the purity data supplied (Chiron Technologies Ltd, Melbourne, Australia). This assay has a detection limit of 1 pmol/l (2 S.D. from zero) and an ED50 of 90 pmol/l. The NT-proCNP assay within- and between-assay coefficients of variation were 6.0 % and 7.9 % respectively, at 19 pmol/l.

**RIA for CNP22**

CNP22 was assayed as described previously [16], except the assay was pre-incubated with antisera for 22 h prior to the addition of [125I]-labelled CNP (8000 c.p.m.) and incubated for a further 24 h at 4 °C. All plasma samples from mother and cord were measured in the one assay, as were cord and placental tissues from the same pregnancy.

**Statistical methods**

Data are means ± S.E.M. Hormone data were log-transformed to satisfy parametric assumptions prior to being analysed by repeated measure ANOVA to determine whether source of plasma (maternal or umbilical cord blood) or type of pregnancy (normal or pre-eclamptic) influenced hormone levels.

**RESULTS**

**Maternal and umbilical cord plasma levels of CNP and NT-proCNP**

Umbilical cord plasma concentrations of both CNP and NT-proCNP were significantly elevated ($P < 0.001$)
compared with maternal levels (Figure 1). This was particularly marked for NT-proCNP with cord plasma concentrations being 10.7 ± 1.1-fold higher than maternal levels in the normotensive group, whereas CNP levels were only elevated 2.2 ± 0.3-fold in cord compared with maternal plasma (Table 2). The ratio of NT-proCNP/CNP in cord plasma (75.2 ± 8.9) was considerably greater than that seen in maternal plasma (14.6 ± 1.4; \( P < 0.001 \)).

Although the hormone levels in the normotensive and pre-eclampsia study groups were similar, the ratio of plasma umbilical cord NT-proCNP to maternal concentrations was significantly greater (\( P = 0.03 \)) in the normotensive group (Table 2). A significant plasma source (maternal or umbilical) by study group (normotensive or pre-eclampsia) interaction for NT-proCNP was identified between the two study groups (\( F = 5.8, P = 0.025 \)). The mean NT-proCNP concentrations in umbilical plasma were greater in the normotensive compared with the pre-eclampsia group (246 ± 17 compared with 203 ± 14 pmol/l respectively), whereas maternal NT-proCNP levels were elevated in the pre-eclampsia group (32.8 ± 3.7 compared with 24.3 ± 1.8 pmol/l). No significant plasma source by study group interaction was observed for CNP.

Table 2  Maternal and umbilical cord plasma levels of CNP and NT-proCNP at delivery after normotensive pregnancies (\( n = 10 \)) or pregnancies complicated by pre-eclampsia (\( n = 13 \))

<table>
<thead>
<tr>
<th></th>
<th>Normotensive gestation</th>
<th>Pre-eclamptic gestation</th>
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<tbody>
<tr>
<td></td>
<td>Umbilical cord plasma</td>
<td>Maternal plasma</td>
</tr>
<tr>
<td>CNP</td>
<td>3.6 ± 0.4*</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>NT-proCNP</td>
<td>246 ± 17*</td>
<td>24.3 ± 1.8</td>
</tr>
<tr>
<td>NT-proCNP/CNP</td>
<td>75.2 ± 8.9†</td>
<td>14.6 ± 1.4</td>
</tr>
<tr>
<td>Cord/maternal</td>
<td>2.2 ± 0.3</td>
<td>10.7 ± 1.1†</td>
</tr>
</tbody>
</table>

Figure 2 Tissue levels of (a) NT-proCNP and (b) CNP in umbilical cord (closed bars) and placental tissue (open bars) extracts obtained at delivery from normotensive or pregnancies complicated by pre-eclampsia

Data (means ± S.E.M.) are expressed as fmol of hormone/g of wet weight tissue.

Placental tissue levels of CNP and NT-proCNP

Concentrations of CNP (Figure 2) were slightly, but significantly, lower in umbilical cord tissue compared...
Figure 3 SE-HPLC of extracts of (a) pooled neonatal umbilical cord plasma and (b) matching pooled maternal plasma
Column void volume (Vo) and elution positions of molecular mass markers are shown by arrows. Immunoreactive (ir) NT-proCNP (●) and CNP (○) profiles are shown.

with placental tissue (4.4 ± 0.3 compared with 5.7 ± 0.3 fmol/g; \( P = 0.004 \)). By contrast, NT-proCNP concentrations were much greater in umbilical tissue than placental tissue (164 ± 5 compared with 104 ± 12 fmol/g; \( P < 0.001 \)). No significant differences in CNP or NT-proCNP concentrations in placental or umbilical cord tissues were seen between the normotensive and pre-eclampsia groups.

Maternal and fetal circulating forms
Maternal and cord plasma from three normotensive women were each pooled and extracted on Sep Pak C\(_{18}\) cartridges prior to SE- and RP-HPLC. A single large peak of NT-proCNP immunoreactivity was seen in the cord plasma SE-HPLC profile (Figure 3a), which eluted at fraction 29 (molecular mass from SE-HPLC = 5 kDa). The major maternal NT-proCNP immunoreactive peak eluted at a similar position (Figure 3b). CNP immunoreactivity in all these fractions was less than the assay detection limit.

Subjecting umbilical cord plasma extracts to RP-HPLC revealed two immunoreactive NT-proCNP peaks (fractions 42 and 44; Figure 4a) and two immunoreactive CNP peaks (fraction 46 and 48; Figure 4b). Synthetic CNP-22 and CNP-53 were shown to elute at fractions 47 and 48 respectively. RP-HPLC of maternal plasma also revealed immunoreactive NT-proCNP peaks at fractions 42 and 44 (Figure 4c). No immunoreactive CNP was detected in the maternal fractions.

DISCUSSION
CNP, acting via the guanylate cyclase receptor NPR-2, has multiple biological actions under experimental conditions, including vasodilation [17], inhibition of VSMC [9] and hepatic myofibroblastic stellate cell growth [18]. It is expressed in a wide variety of tissues, including reproductive tissues. In the female, mRNA levels in uterine and ovary tissue are elevated at proestrus.
[8] and, in placenta, strong CNP mRNA expression was seen in the decidua basalis around large maternal blood vessels [19]. In the male, high concentrations of CNP are present in seminal plasma [20] and in the Leydig cells of the testis [7]. Despite these findings, the function of CNP remains unclear, although a role in the regulation of vascular tone [21] has been suggested.

In normal adult subjects, CNP levels in the circulation are often at, or below, the limits of detection due to rapid metabolism by clearance receptors and the enzyme neprilysin. In contrast, NT-proCNP circulates at higher concentrations thus providing a measurable index of CNP production [13]. In the present study, we have documented levels of both CNP and NT-proCNP in plasma from maternal and placental cord blood at delivery in women who were normotensive or had their pregnancy complicated by pre-eclampsia.

Using SE-HPLC we have identified for the first time in umbilical cord plasma a 5 kDa N-terminal fragment of proCNP that is identical or closely similar to NT-proCNP(1–50). A similar sized peptide was also identified in maternal plasma, suggesting that the processing of maternal and fetal proCNP is identical (Figure 3). There was no evidence that proCNP (molecular mass, 10.9 kDa) circulates in the fetus. RP-HPLC revealed the 5 kDa peptide comprised two distinct molecular forms of NT-proCNP in both fetal and maternal plasma (Figures 4a and 4c). The precise nature of these forms remains to be determined. Plasma concentrations of NT-proCNP in umbilical cord blood sampled immediately after delivery were 10-fold higher than the corresponding maternal venous levels. The small, but significantly higher, levels of CNP in cord compared with maternal plasma (3.6 compared with 1.8 pmol/l respectively) closely reflects the fetal–maternal gradient described previously by Stepan et al. [14]. What is striking about the present findings is the large increase in the NT-proCNP/CNP ratio in the fetus when compared with that found in normal adult or maternal plasma, suggesting altered clearance of these hormones in the fetus. Conceivably, CNP synthesis and clearance (uptake of CNP but not NT-proCNP by clearance receptors and metabolism of CNP by neprilysin) in the fetus are both greatly increased, or (less likely) the half-life of NT-proCNP is selectively prolonged, prior to birth. The alternative, that placental tissue contributes selectively to NT-proCNP levels in cord blood, seems unlikely, since the molar concentration of NT-proCNP was lower in placental tissue extracts than cord plasma. Further work is clearly required to determine the source of NT-proCNP in fetal tissues.

No significant differences in concentrations of CNP in umbilical cord or maternal plasma between pre-eclamptic and normal women were seen. This result supports the finding of a previous study [22] in which plasma concentrations of CNP during gestation (26–39 weeks) were not significantly different between women with normal pregnancies and those complicated by gestational hypertension and pre-eclampsia. However, when plasma levels of NT-proCNP were analysed, a statistically significant interaction between plasma source (maternal or fetal) and gestational group (normotensive or pre-eclamptic) was identified. The mean maternal plasma NT-proCNP concentrations were greater in the pre-eclampsia group, whereas the converse was observed in the umbilical cord plasma. It is possible that these differences may be due to the shorter gestational age and/or lower birth weight seen in the pre-eclampsia group (Table 1). The biological significance of these findings remains to be explored.

In summary, we have shown that NT-proCNP(1–50) circulates in the fetus at greatly increased levels when compared with CNP, which is minimally elevated above maternal levels. The findings are consistent with both increased tissue production of CNP and its clearance by neprilysin and/or specific clearance receptors, which are unlikely to bind NT-proCNP.

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