Downregulation of Apelin in the Human Placental Chorionic Villi from Preeclamptic Pregnancies

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Running Head: Apelin peptides in human placenta in preeclampsia

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Abstract

The role of the endogenous apelin system in pregnancy is not well understood. Apelin’s actions in pregnancy are further complicated by the expression of multiple forms of the peptide. Using radioimmunoassay (RIA) alone, we established the expression of apelin content in the chorionic villi of preeclamptic (PRE) and normal pregnant women (NORM) at 36-38 weeks of gestation. Total apelin content was lower in PRE compared to NORM chorionic villi (49.7±3.4 vs. 72.3±9.8 fmol/mg protein, n=20-22) and was associated with a trend for lower preproapelin mRNA in the PRE. Further characterization of apelin isoforms by HPLC-RIA was conducted in pooled samples from each group. The expression patterns of apelin peptides in NORM and PRE villi revealed little or no apelin-36 or apelin-17. Pyroglutamate apelin-13 [(Pyr1)-apelin-13] was the predominant form of the peptide in NORM and PRE villi. ACE2 activity was higher in PRE villi (572.0±23.0 vs. 485.3±24.8 pmol/mg/min, n=18-22). Low dose of Ang II (1nM; 2 hours) decreased apelin release in NORM villous explants that was blocked by the AT1 receptor antagonist losartan. Moreover, losartan enhanced apelin release above the 2-hour baseline levels in both NORM and PRE villi (p<0.05). In summary, these studies are the first to demonstrate the lower apelin content in human placental chorionic villi of PRE subjects using quantitative RIA. (Pyr1)-apelin-13 is the predominant form of endogenous apelin in the chorionic villi of NORM and PRE. The potential mechanism of lower apelin expression in the PRE villi may involve a negative regulation of apelin by Ang II.

Keywords: Apelin-13, apelin receptor, ACE2, pregnancy, explant
**Introduction**

Preeclampsia is a pregnancy-induced hypertensive disorder that occurs in 5 to 7% of all pregnancies worldwide (54). It is associated with maternal perinatal morbidity and mortality and a high risk of premature birth and fetal growth restriction. Despite continued research, the pathogenesis of preeclampsia is still unknown. The endogenous apelin system is an emerging target for the regulation of cardiovascular homeostasis (22); however, its role in pregnancy is not well understood. The levels of preproapelin mRNA are widespread in human tissues, and high levels were identified in the placenta (38), suggesting a possible placental origin of apelin in pregnancy. Apelin may have a paracrine role in human chorionic villi as it has been identified in cytotrophoblasts, syncytiotrophoblasts, and in fetal endothelial cells (13; 15). A positive correlation between maternal and fetal plasma apelin was observed in full-term normal pregnancies (37). Moreover, a transplacental transfer of apelin with potential impact on fetal growth was suggested (37). Apelin is a ligand for human G-protein coupled receptor, APJ (40). The role of APJ in embryonic development was shown by the existence of cardiovascular developmental defects in APJ deficient mice (11). In addition, a small litter size and a significant loss of homozygous animals were noted in these animals (11). Apelin/APJ system has pleiotropic roles in cardiovascular physiology (40). Apelin’s ability to lower blood pressure was demonstrated chronically in a mouse model of atherosclerosis and acutely in patients with heart failure (12; 22; 31; 32). Moreover, apelin induces cardiac contractility (48) and vessel formation (25) and suppresses aortic inflammation via downregulation of interleukin-6 and TNF alpha mRNA (33). Because of the cardiovascular protective actions of apelin in addition to its role in the regulation of fluid homeostasis and metabolic functions, the endogenous apelin/APJ system may be important for adaptation to pregnancy and for regulation of fetal growth (51).
The regulation of apelin peptides in the placenta is unknown. Evidence supports a counter-regulatory role for apelin in relation to the renin-angiotensin system (RAS) through the functional interactions between apelin and angiotensin II (Ang II) (16; 21; 43). Ang II and apelin have opposing actions on blood pressure regulation, vascular tone, inflammation and fluid homeostasis, factors known to be important in pregnancy (52);(17; 51). Although the systemic RAS is down-regulated in preeclampsia (34), the contribution of local uteroplacental Ang II/angiotensin II receptor 1 (AT1R) to the pathogenesis of preeclampsia has been suggested (1-4; 7; 17-19). Anton and Brosnihan demonstrated the up-regulation of vasoconstrictor and pro-inflammatory peptide Ang II and AT1R in the chorionic villi of women with preeclampsia at term (1), suggesting a paracrine role of local Ang II/AT1R in the placenta. Moreover, preeclampsia in female rats with overexpression of human angiotensinogen gene mated to male rats with overexpression of human renin gene (17), exhibits upregulated placental Ang II, underscoring the contribution of the local uteroplacental RAS to preeclampsia. It is possible that Ang II may down-regulate apelin expression or its release from the preeclamptic chorionic villi and therefore interfere with its action in preeclampsia.

The processing of the apelin precursor is complex and may yield a number of bioactive peptides. The peptide is synthesized as a 77 amino acid pre-prohormone that is processed into a 55 residue intermediate and then to a 36 residue peptide, apelin-36; further processing of apelin-36 generates apelin-17, apelin-13 and apelin-12 (5; 31). In addition, the prohormone convertase PCSK3 or furin can hydrolyze the apelin propeptide directly to apelin-13 in adipocytes (42). In contrast, angiotensin converting enzyme 2 (ACE2) acts on the C-terminus of apelin-13 and apelin-36 to cleave the Pro-Phe bond with high catalytic efficiency (42) and constitutes a pathway for apelin degradation. Additionally, (Pyr1)-apelin-13 is a posttranslationally modified
form of apelin-13 containing the pyroglutamate group at the N-terminus of the peptide. The
twelve C-terminal amino acid peptide is considered the minimal required sequence for apelin
biological activity (40). Presently, it is not known whether differences exist in the expression of
the various molecular forms of apelin in the human placenta or that the expression patterns of
apelin forms are altered in preeclampsia. In this study we compared the immunoreactive apelin
content in preeclamptic chorionic villi to villi obtained from women with normal pregnancy
using a quantitative radioimmunoassay (RIA) that recognizes multiple forms of apelin. Using
reverse-phase high performance liquid chromatography (HPLC) we established the expression
patterns of the apelin peptides in normal and preeclamptic villi. In addition, we determined
whether Ang II influences apelin release as one of the mechanisms of apelin regulation in the
human chorionic villi.

**Materials and Methods**

**Human Subjects:** Group 1 – normal pregnancy group (n=22) - normotensive pregnant
subjects (systolic blood pressure <140/90 mmHg) in their third trimester of pregnancy with no
history of chronic blood pressure elevation and the absence of proteinuria (4). Group 2 –
preeclamptic group (n=20) – women with new-onset hypertension (>140/90 mmHg) and
proteinuria (≥ 300 mg/24 hours or 2+ or more protein on a random sample on 2 or more
occasions 4 hours apart). Consent forms were signed by all participants at the time of their
enrollment in the study. Systolic and diastolic blood pressures and proteinuria were recorded at
the time of delivery. All pregnant women were nulliparous, and underwent natural delivery or
cesarean section. All subjects were free of any known pre-existing cardiovascular, endocrine, or
connective tissue diseases. The study was approved by the institutional review boards (IRB) at both Wake Forest School of Medicine and Forsyth Medical Center (BF04-222) (4).

**Human placental chorionic villous tissue collection:** Immediately after delivery (within 15 min), the whole placenta was collected on ice, and tissue sections were taken from the center of the placenta, near the umbilical cord attachment site. For each tissue section, the maternal basal plate and fetal membranes were removed so that only the fetal villous tissue was present; areas of necrosis or tissue damage were avoided. Each piece of tissue was either 1) snap frozen in liquid nitrogen and stored at −80°C for future experiments (4), or 2) several small pieces were placed into a cassette and fixed in 10% neutral buffered formalin for 24 hours for immunohistochemistry. The tissue was taken as part of a previously published study (4); the additional aliquots used for the study were kept frozen at -80°C until analyzed.

**Human placental chorionic villous explant culture:** Chorionic villi were collected from women with normal and preeclamptic pregnancy using the same criteria as described above. After 2 hours of equilibration period, chorionic villi (1 gram of tissue) were exposed to 1 nM of Ang II alone or in combination with losartan (1µM), an antagonist for AT₁R for 2 hours under 5% N₂ (pO₂ in media = 60 mm Hg) supplemented with 5% CO₂ as previously described by our group (45). The unstimulated release of apelin from chorionic villi for 2 and 16 hours was also determined (45). The media was collected and frozen at -80°C until assayed for apelin release. Apelin release was determined using the RIA (Phoenix Pharmaceuticals, Burlingame, CA) as described below.

**Radioimmunoassay:** An apelin RIA was performed according to the manufacturer’s instructions (Phoenix Pharmaceuticals, Burlingame, CA); however, the tissue extraction was modified. For the detection of total apelin content in the chorionic villi, individual samples from
normal and preeclamptic pregnant women were tested. The RIA assay recognizes the C-terminal peptide and therefore detects all forms of apelin including -12, -13, (Pyr¹)-13, -17, -36, and the prepropeptide (23-77) and is designated immunoreactive apelin with direct RIA assessment. The minimum detectable level of the RIA is 4 picogram per tube. Tissue homogenization conditions were developed to coincide with the HPLC separation; the homogenization procedure was different from that recommended for the RIA kit. Specifically, tissues (100 mg) were homogenized in 10 ml acid ethanol (80% vol/vol 0.1 N HCl) solution containing the peptidase cocktail of inhibitors for angiotensin peptides except without the rat renin inhibitor (10). The supernatant of the tissue samples was extracted using C-18 Sep-Pak columns activated with 5 ml of acid methanol followed by 20 ml of 0.1% heptafluorobutyric acid (HFBA). The sample was applied and the column was washed with 10 ml 0.1% HFBA. The column was then washed with 5 ml H₂O and then eluted with 3.3 ml of acid methanol (0.1% HFBA and 80% methanol). The sample was then dried in a Savant vacuum centrifuge (Farmingdale, NY) and reconstituted for the RIA (41). Radiolabeled apelin was added to the sample when homogenized and followed through the extraction process. The recovery of radioactive apelin was between 60-65%. The total apelin concentrations were corrected for recoveries.

**HPLC analysis:** HPLC and RIA were applied to resolve the following apelin peptides: -12, -13, (Pyr¹)-apelin-13, -17 and -36 using the appropriate standards (apelin-12: Anaspec, Fremont, California; apelin-13: Cayman Chemical, Ann Arbor, Michigan; (Pyr¹)-apelin-13: Anaspec, Fremont, California; apelin-17: Abcam, Cambridge, MA; apelin-36: Anaspec, Fremont, California). The analysis of apelin molecular forms was performed using placental samples of 200 mg each that were randomly pooled from either four normal pregnant or four preeclamptic women collected at the time of delivery. The pooled samples were initially
fractionated on a Shimadzu Prominence HPLC with a Nova Pak C18 column (2 x 150 mm, Waters, Bedford, MA) using 0.1% HFBA (A) and 80% acetonitrile (B) solvent system at a flow rate of 0.35 ml/min and a gradient of 30-50% B over 20 minutes. Further separation of lower molecular weight forms of apelin was achieved using a gradient of 32-40% B over 25 minutes on the same HPLC system. Fractions were collected at 1 min intervals, completely evaporated and analyzed by the apelin RIA. Products were identified by comparison to apelin standards. To determine the elution time of oxidized apelin peptides, 1 mg/ml of (Pyr1)-Apelin-13 or apelin-12 was incubated with 0.05% H₂O₂ for 15 minutes, and then run on HPLC.

**Real-time qPCR:** Quantitative real-time reverse transcriptase, real-time polymerase chain reaction (qPCR) was performed to assess preproapelin or apelin receptor mRNA concentrations in the chorionic villi obtained from the normotensive and preeclamptic women as previously described (57). All probes were purchased from Life Technologies (Grand Island, NY, USA). 18S ribosomal RNA (rRNA), amplified using the TaqMan Ribosomal RNA Control Kit (Life Technologies), served as an internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product is first detected, and expressed as the ratio of target/control.

**Immunohistochemistry:** Tissues were fixed in formalin and 70% ethanol, embedded in paraffin and cut into 5-µm sections. Immunostaining was performed using the Avidin Biotin Complex (ABC) method with 0.1% diaminobenzene solution used as the chromogen as described previously (56). Antigen retrieval treatment with sodium citrate buffer (pH 6.0) was applied at 90-95°C for 30 min. Non-specific binding was blocked in a buffer containing 10% normal goat serum, 1% Triton-X in PBS for 30 min. Chorionic villi sections were incubated with the rabbit polyclonal anti-APJ primary antibody (dilution: 1:1000; Millipore, Merck KGaA,
Darmstadt, Germany) and secondary biotinylated goat anti-rabbit antibody (dilution: 1:400; Vector Laboratories, Burlingame, CA, USA). APJ distribution was examined under a light microscope and photographed with a QImaging Retiga 1300R CCD digital camera and SimplePCI (Version 6) software.

**Western blot hybridization:** Placental (100 µg of the total protein) homogenates were prepared for the detection of APJ receptor by Western blot hybridization as described (55). Rabbit affinity purified polyclonal anti-APJ antibody recognizing APJ at 62 kilodalton (kDa) (Millipore, Merck KGaA, Darmstadt, Germany), was applied overnight at 1:600 dilution at 4ºC. The data were quantified using densitometry (MCID software); the results were expressed as arbitrary units of intensity, and normalized to β-actin intensities as described (55). Mouse cerebellum tissue lysate was used as a positive control.

**ACE2 activity assay:** An ACE2 fluorescence assay was performed according to Vickers et al. (53) with modifications. Reaction mixtures containing the substrate, 50 mM 7-methoxycoumarin-4-acetyl-alanine-proline-lysine-(2,4dinitrophenyl)-OH, or human placenta, and 10 mM HEPES, pH 7.0, with 1.0 M NaCl were incubated at 37ºC for 60 min with inhibitors to block residual ACE, nephrilysin, or carboxypeptidase A activity. A second set of reactions contained the selective ACE2 inhibitor C16, a generous gift of the Millennium Corporation, to ensure that the measured enzyme activity was ACE2. The reaction was terminated by adding 0.2% trifluoroacetic acid; the fluorescence was quantified in a Perkin Elmer LS 50B fluorometer (excitation – 320 nm; emission – 405 nm).

**Statistics:** Comparisons of apelin content, APJ protein, preproapelin or APJ mRNA levels between the normal pregnancy and preeclamptic groups were performed using unpaired Student’s t-test. The release of apelin into the media over time (0, 2, and 16 hours) was analyzed
using two-way analysis of variance (ANOVA) followed by the Bonferroni’s post-test (GraphPad Software, San Diego, CA). Two-way ANOVA with Bonferroni’s post-test was also used to detect differences in apelin release among the baseline (0 hour), Ang II treatment alone or Ang II plus losartan. A p value less than 0.05 was considered statistically significantly different. All data are presented as mean \( \pm \) SEM except the data on Table 1 that are shown as mean \( \pm \) standard deviation of the mean (SD).

Results

A summary of the patient population characteristics is shown in Table 1. There were no differences in the biological age between women with normal and preeclamptic pregnancies. Preeclamptic women had pregnancies that were on the average 1.6 weeks shorter; their babies were 16% smaller. The majority of women in the preeclamptic group were diagnosed with a late-onset form of preeclampsia (4). Preeclamptic subjects had 17% higher body mass index compared to normal pregnancy group. Systolic and diastolic blood pressures were greater in the preeclamptic group versus normal pregnancy group (Table 1).

As shown on Figure 1A, the content of total apelin determined by direct RIA was significantly lower in preeclamptic chorionic villi, compared to normal villi \( (n=20-22, p<0.05) \). Tissue mRNA levels of apelin prepropeptide tended to be lower in preeclamptic chorionic villi \( (n=15-18, p<0.09) \) (Figure 1B), although this difference did not attain statistical significance. However, the protein and mRNA levels of the APJ receptor were not different between the two groups (Figure 1C-D). Figure 1E shows the immunocytochemical distribution of APJ receptor in normal and preeclamptic chorionic villi: the APJ receptor was identified in syncytiotrophoblasts depicted by black arrow, cytotrophoblasts (black arrowhead) and endothelial cells of fetal
capillaries (red arrow) of human chorionic villi. The distribution pattern of APJ immunostaining appeared to be similar in normal and preeclamptic chorionic villi. Syncytiotrophoblasts are lining the lacuna system. For the most part, these cells had strong cytoplasmic staining. The fetal blood vessels endothelium staining was also positive. Villi are supported by mesenchymal cells that are responsible for producing the intercellular matrix. Both the endothelium (Figure 1E) and mesenchymal cells (not shown) were positive for APJ; however, the staining appears to not be as strong as that observed in the syncytiotrophoblasts.

The apelin forms in the placenta were then fractionated by HPLC separation and RIA detection of the resultant fractions. The initial HPLC method resolved apelin-36 and apelin-17 from the smaller isoforms; however, this gradient did not distinguish apelin-12, apelin-13 and (Pyr\(^1\))-apelin-13 (Figure 2). The HPLC assessment of pooled samples from normotensive and preeclamptic tissue revealed that the smaller isoforms including apelin-12, apelin-13 and (Pyr\(^1\))-apelin-13 were the primary immunoreactive peaks with little or no evidence of endogenous apelin-17 and apelin-36 (Figure 2). To better resolve the smaller apelin forms, a shallower gradient was employed for the HPLC fractionation that fully resolved (Pyr\(^1\))-apelin-13 and partially resolved apelin-12 and apelin-13; the larger forms (apelin-36 and apelin-17) were retained to a far greater extent and these fractions were not collected for RIA measurement (Figure 3). This second HPLC analysis revealed that the two major apelin peaks were (Pyr\(^1\))-apelin-13 (14 min elution) and oxidized (Pyr\(^1\))-apelin-13 (10 min elution) in both preeclamptic and normal villi (Figure 3B, upper insert). We also noted two immunoreactive peaks corresponding to apelin-13 (17 min) and apelin-12 (18 min) in pooled tissues from both preeclamptic and normal chorionic villi (Figure 3A, B, and C). Finally, an additional
immunoreactive peak that eluted at 15 min appears to be the oxidized form of apelin-12 and was also evident in both groups (Figure 3B, lower insert).

Since ACE2 may contribute to apelin processing, we utilized a fluorescent assay to quantify activity in the villi. In Figure 4 we show that the preeclamptic chorionic villi exhibit significantly higher ACE2 activity as compared to normal villi (n=18-22, n<0.05).

We then determined the extent that villi may release apelin peptides using an *ex vivo* incubation system coupled to apelin detection by direct RIA. In Figure 5A, we show a significant increase in unstimulated release of apelin from chorionic villous explants from women with normal pregnancy at 2 and 16 hours and preeclamptic pregnancy at 16 hours. As shown on Figure 5B, baseline release of apelin at 2 hours tended to be lower in the preeclamptic villi (p<0.09); a low dose of Ang II (1 nM) significantly inhibited the release of apelin only from the normal villi at 2 hours. Addition of the AT1 receptor antagonist, losartan, abolished the Ang II-induced decrease in apelin release from chorionic villous explants. Moreover, losartan enhanced apelin release above the 2-hour baseline levels in both normal and preeclamptic villi (Figure 5B).

**Discussion**

Preeclampsia is a life threatening disorder specific to pregnancy with no current treatment. The only cure for this disease is delivery of the placenta which is considered the primary source of inflammatory and vasoconstrictor factors to initiate widespread endothelial injury and vasoconstriction, upon release into maternal circulation (35). Understanding molecular factors that lead to preeclampsia could provide a basis for therapeutic interventions that may reduce the incidence of this disease, improve maternal and fetal survival, ultimately leading to improved health of the whole population. The apelin/APJ system is a novel pleiotropic
pathway with a potential for therapeutic targeting in preeclampsia. However, the characterization of apelin actions in pregnancy and preeclampsia is complicated by the existence of multiple forms of the active peptide. To date, the majority of studies on apelin characterization in human placenta are limited to the analysis of peptide expression using immunohistochemical methods or PCR analysis of the precursor protein. However, a complete analysis of the apelin system in human placenta requires a greater resolution of the molecular forms of functional apelin peptides. The use of RIA provides a quantitative analysis of the total apelin levels, while a combined HPLC-RIA method allows for the characterization of the expression patterns of apelin forms in the human placenta.

Several studies have recently investigated the expression of apelin in human placenta. Inzukua et al. reported that apelin mRNA and immunohistochemical peptide levels were lower in placenta from preeclamptic women (20). Our findings agree with this study and demonstrate that total apelin content in the chorionic villi is 30% lower in preeclamptic women as quantified by direct RIA. Moreover, the expression patterns of apelin peptides in the normal and preeclamptic chorionic villi using HPLC-RIA revealed that (Pyr\textsuperscript{1})-apelin 13, apelin 13, apelin 12 and possibly oxidized apelin 12 were the major forms in both normal and preeclamptic chorionic villi. The presence of the pyroglutamate moiety prevents enzymatic breakdown by aminopeptidases, thus preserving the biological activity of the peptide (27). Indeed, this protective group may contribute to the visibly higher (Pyr\textsuperscript{1})-apelin-13 peak in chorionic villi of both normal and preeclamptic women relative to the other molecular forms of apelin. (Pyr\textsuperscript{1})-apelin-13 was identified as the predominant form of apelin in the pooled atrial appendage tissue using HPLC-RIA (36) and in human plasma by mass spectrometry (58). However, it is important to note that the current HPLC analysis of apelin isoforms was performed on a small number of
pooled samples that precludes a statistical analysis of the individual peptide isoforms between
groups. Additional studies are necessary to increase the sample size for the peptide analysis, as
well as achieve a more complete resolution of the apelin forms in the placental tissue. In
addition, a higher body mass index (adiposity) may affect apelin levels in the preeclamptic
women cohort. A higher body mass index is increasingly associated with an augmented risk of
preeclampsia, and it is one of the contributing factors to the development of preeclampsia (9;
14). Adiposity leads to the release of many inflammatory or angiogenic factors and therefore it is
possible that the biochemical profiles of apelin peptides are in part influenced by the metabolic
activity of adipocytes (50).

The blood pressure-lowering actions of apelin-12, apelin-13, and (Pyr1)-apelin-13 are
apparent in different animal models and in patients with chronic heart failure (12; 22; 31; 32;
49). Intravenous infusion of (Pyr1)-apelin-13 reduced blood pressure and peripheral vascular
resistance in heart failure patients (22). Apelin-12 and apelin-13 reduced both systolic and
diastolic blood pressures in anesthetized normotensive Wistar rats and spontaneously
hypertensive rats (SHR) (31; 32). However, the magnitude of the acute apelin-13 effect on blood
pressure response was two-fold higher than apelin-12, suggesting a greater potency of apelin-13
(32). Modification of the carboxyl-terminal phenylalanine (Phe) of apelin-13 inhibited the
hypotensive effect suggesting that Phe is required for the blood pressure-lowering actions of this
peptide (32). Apelin-13 also decreased blood pressure in a mouse model of atherosclerosis when
administered chronically for four weeks (12). Therefore, it is feasible that lower apelin levels in
human preeclamptic placenta may diminish its opposing modulation of vasoconstrictor mediators
which result in an increased blood pressure in preeclampsia. In addition to effects on blood
pressure, (Pyr1)-apelin-13 and other apelin peptides may induce vasodilation or reverse arterial
constriction of multiple vascular beds including mammary, mesentery, coronary, and splanchnic arteries (22; 36). The vasodilatory effects of apelin in vasculature are due to activation of Akt and eNOS to generate nitric oxide (NO) (12; 21; 49; 59). This is consistent with studies showing that apelin-induced endothelium-dependent vasorelaxation is attenuated by co-administration of L-NAME, suggesting an endothelium-derived NO-dependent mechanism (24). However, apelin may also induce vasoconstriction via its direct effects on vascular smooth muscle cells (24; 26; 28; 39). Studies investigating vascular actions of apelin in pregnancy are necessary to elucidate the role and mechanisms of action of this peptide in maternal and fetal vasculature.

We noted additional immunoreactive peaks on chromatogram that did not correspond to any of the apelin standard peptides. Although the exact mechanism for formation of these additional peaks is presently unknown, increased oxidation during the tissue processing protocol may contribute to their formation. As in our study, an oxidized form of (Pyr1)-apelin-13 was also detected in the heart tissue by HPLC (36) and this peak eluted at an earlier time point compared to that of apelin-13 or (Pyr1)-apelin-13.

ACE2, an enzyme of the renin-angiotensin system that degrades angiotensin II to angiotensin-(1-7) and angiotensin I to angiotensin-(1-9), is the only known enzyme that can degrade apelin-36 and apelin-13 with high catalytic efficiency (53). Once phenylalanine on the C-terminus is released by ACE2 (53), the peptide may not be recognized by antibodies used for the apelin RIA. It is possible that ACE2 processes apelin-12 and apelin-13 peptides to a different extent than (Pyr1)-apelin-13. Although we did not directly test ACE2 degradation of apelin forms in human chorionic villi, our data suggest that relatively lower levels of apelin content in preeclamptic chorionic villi may reflect higher ACE2 activity.
In regards to the APJ receptor, our data also support other studies showing the APJ receptor expression in syncytiotrophoblasts, cytotrophoblasts, and endothelial cells of fetal capillaries of human chorionic villi (13; 15), suggesting a paracrine role of apelin in the uteroplacental unit. The angiogenic properties of apelin as well as apelin/APJ activation of cell migration and proliferation may be beneficial during the development of the placenta and embryogenesis (23; 30). APJ mRNA or protein expressions were not different between studied groups in our patient cohorts. Furuya et al. reported higher APJ mRNA levels in the placentas obtained from a cohort that included women with gestational hypertension without preeclampsia combined with women with preeclampsia as compared with normal pregnancy (15). Because of their combined group analysis, it is difficult to compare the results of their study with ours. In addition, a decreased APJ immunostaining was described in the early-onset preeclamptic villi at the third trimester compared with normal placenta (13; 15).

Plasma apelin measured at delivery was reported to be lower (6), not different (29), or higher (20) in women with preeclampsia compared with women with normal pregnancy. It is known that apelin levels are influenced by endogenous enzyme inhibitors that are present in the plasma (58). We note that these studies did not attempt to fractionate the various molecular forms of apelin. Aminopeptidases, serine proteases, and to a lesser extent, carboxypeptidases are likely responsible for the processing of apelin peptides to lower molecular weight products or inactive apelin fragments in human plasma (58). Differences in patient population characteristics, the type of assay used to quantify apelin, and sample collection conditions may account for the discrepancy of these results. In addition, maternal plasma levels are dependent on placental levels of apelin, as the removal of half of the fetomaternal unit increases maternal plasma levels by 23 percent (51). This observation would be consistent with degradative
enzymes in the placenta that may influence the various forms of apelin in the maternal circulation.

Finally, apelin and Ang II are counter-regulators of the cardiovascular system (12; 43; 44; 47). Therefore, it is possible that Ang II can interfere with apelin formation or apelin actions in the placenta. Our data show that apelin release from the chorionic villi was decreased by the administration of Ang II, suggesting a potential interaction between apelin and Ang II. Moreover, losartan enhanced apelin release above the baseline levels. Since the actions of AT2 receptor (AT2R) could be unmasked following the AT1R blockade (8; 46), it is possible that AT2R contributes to apelin release from chorionic villi. AT1R is the predominant angiotensin receptor in normal and preeclamptic placental chorionic villi (4). We found that the actions of Ang II to downregulate apelin release from chorionic villi were AT1R dependent. In contrast Ang II enhanced apelin secretion in 3T3-L1 adipocytes by an AT1R Ca2+/PKC or MAPK-dependent pathway, while AT2R activation inhibited apelin release via a PKA/cAMP and cGMP pathways (50). It is possible that regulation of apelin by Ang II is tissue or cell specific which may account for the differences seen between these and our experiments.

In summary, our studies are the first to demonstrate lower apelin content in the human placenta using quantitative radioimmunoassay in normal and preeclamptic women. In both normal and preeclamptic chorionic villi, (Pyr1)-apelin 13 was the predominant form in the chorionic villi. Moreover, the potential mechanism of lower apelin expression in the PRE villi may involve a negative regulation of apelin by Ang II. Additional studies are warranted to quantitatively examine the expression patterns of apelin peptides and to assess the functional significance of apelin peptides in preeclampsia.
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Figure Legends:

**Figure 1.** The characterization of apelin content and receptor (APJ) in human chorionic villi obtained from women with preeclampsia versus women with normal pregnancy. Figure 1A: The content of immunoreactive apelin in the chorionic villi, *p<0.05* vs. normal pregnancy, individual samples of n=20-22. Data are mean ± SEM. Abbreviations: Norm, normal pregnancy; PRE, preeclamptic pregnancy.

Figure 1B: The mRNA levels of preproapelin in the chorionic villi. The gene expression data are expressed as the ratio of apelin to 18S rRNA; p<0.09, n=15-18.

Figure 1C: Protein levels of APJ determined by western blot analysis in the chorionic villi, n=5 in each group. Representative bands for APJ and β-actin are shown.

Figure 1D: The mRNA levels of APJ in the chorionic villi; n=16-18.

Figure 1E: The immunoreactive distribution of APJ in the chorionic villi: APJ was identified in syncytiotrophoblasts (black arrow), cytotrophoblasts (black arrowhead) and endothelial cells of fetal capillaries (red arrow). Magnification: 40x.

**Figure 2.** HPLC analysis of apelin forms in the human chorionic villi in pooled samples from women with normal or preeclamptic pregnancy. The pooled samples were fractionated using 0.1% heptafluorobutyric acid (A) and 80% acetonitrile (B) solvent system at a flow rate of 0.35 ml/min and gradient conditions of 30-50% B in 20 min. Data are reported as pg/tube; a pooled sample from each group was obtained by combining 4 tissue samples from each group. A. Standard profile. B. Profile in normal placenta. C. Profile in preeclamptic placenta

**Figure 3.** HPLC analysis of lower molecular weight apelin forms in pooled samples from women with normal or preeclamptic pregnancy. Gradient conditions were 32-40% B over 25 minutes at 0.35 ml/min (Panels A-C). The peak at 10 minutes is consistent with oxidized form of
(Pyr\textsuperscript{1})-Apelin-13 (Panel B, upper insert), and the 15 minute peak is consistent with the oxidized form of Apelin-12 (Panel B, lower insert). Products were identified by comparison to apelin standards. A. Standard profile. B. Profile in normal placenta with inserts demonstrating the profile of the oxidized forms. C. Profile in preeclamptic placenta.

Data are reported as pg/tube; a pooled sample from each group was obtained by combining 4 samples from each group.

**Figure 4.** ACE2 enzyme activity in human chorionic villi obtained from women with preeclampsia versus women with normal pregnancy. Data are mean ± SEM, *p<0.05 vs. normal pregnancy; individual samples of n=18-22. Abbreviations: Norm, normal pregnancy; PRE, preeclamptic pregnancy.

**Figure 5.** Time course (Panel A) and the effect of Ang II alone or a combination of Ang II with losartan (Panel B) on apelin release into conditioned media from placental chorionic villous explants isolated from women with normal or preeclamptic pregnancy. The data are expressed as pg of apelin per milliliter, n=3-9. *p<0.001 vs. Norm 0 h; #p<0.0001 vs. PRE 0 h; ^p<0.05 vs. Norm baseline (BL) at 2 h; &p<0.001 vs. Norm Ang II; γp<0.01 vs. PRE Ang II; πp<0.05 vs. PRE BL at 2 h. Abbreviations: Ang II, angiotensin II; LOS, losartan; h, hours.
Table 1. Patient Population Profile

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Norm (n=22)</th>
<th>PRE (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.6±1.1</td>
<td>24.3±1.3</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>31.1±1.3</td>
<td>36.4±2.1*</td>
</tr>
<tr>
<td>Birth Weight (g)</td>
<td>3218.0±141.6</td>
<td>2699.9±177.6*</td>
</tr>
<tr>
<td>Gestational Age (weeks)</td>
<td>38.2±0.6</td>
<td>36.6±0.6*</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>144.2±3.8</td>
<td>173.0±3.1*</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>80.5±2.3</td>
<td>106.4±1.6*</td>
</tr>
<tr>
<td>Proteinuria (0-4+)</td>
<td>None</td>
<td>&gt;1+</td>
</tr>
</tbody>
</table>

Abbreviations: Norm, normal pregnancy; PRE, preeclamptic pregnancy.

Data are mean±SD.
A

Apelin Standards

(Pyr$^1$)-Apelin-13
Apelin-13
Apelin-12

Normal Placenta

(Pyr$^1$)-Apelin-13
Ox-(Pyr$^1$)-Apelin-13
Ox-Apelin-13
Ox-Apelin-12

Preeclamptic Placenta

Ox-(Pyr$^1$)-Apelin-13
(Pyr$^1$)-Apelin-13
Apelin-13
Apelin-12
ACE2 Activity

pmol/mg/min

Norm  PRE

*
**Time Course**

A

![Bar chart for Time Course](image)

- **Norm**
  - 0 h
  - 2 h
  - 16 h

- **PRE**

  * * *

**2 Hours**

B

![Bar chart for 2 Hours](image)

- **BL**
- **Ang II**
- **Ang II+LOS**

- **NORM**
- **PRE**

^ & γπ