Maternal Apelin Physiology during Rat Pregnancy: The Role of the Placenta

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ABSTRACT

Objective: Apelin is a multifunctional peptide which is catabolized by the angiotensin-converting enzyme-related carboxypeptidase-2 (ACE2). The peptide is well known for its hemodynamic effects and its role in energy and fluid homeostasis. Pregnancy is a state of dramatically altered maternal homeodynamics and metabolism, but the role of apelin is unknown. To gain further insight in apelin physiology, we investigated relative tissue expression, plasma clearance and metabolic pathways of apelin in pregnant rats.

Methods: We measured maternal plasma apelin levels throughout normal rat gestation and examined relative apelin gene expression in several tissues, including the placenta. We documented apelin clearance using radiolabeled apelin and assessed maternal plasma levels in rats that underwent surgical reduction of the fetoplacental mass, thereby further examining the role of the placenta in apelin clearance. Finally, we localized apelin and ACE2 in the placenta and mesometrial triangle using immunohistochemistry.

Results: Maternal apelin plasma concentrations dropped by 50% between mid- and late gestation. Apelin expression was comparable between non-pregnant and late-pregnant rats in non-reproductive tissues. The placenta showed low apelin gene expression compared to brain tissue. Apelin clearance was enhanced in term gestation as evidenced by a steeper decline of the slow phase of the elimination curve of radiolabeled apelin. Compared to sham-operated dams, maternal plasma apelin was raised by 23% in late-pregnant rats in which half of the fetoplacental units were removed at day 16 of gestation. ACE2 mRNA expression was detectable in late- but not mid-pregnancy placental tissue; immunohistochemically, ACE2 was primarily localized in the smooth muscle layer of fetal arterioles in the labyrinth.

Conclusion: Maternal circulating apelin drops considerably between mid- and late pregnancy owing to faster clearance. The current data suggest a role for placental ACE2 in the accelerated apelin metabolism.

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1. Introduction

Apelin, a 36 aminoacid peptide (M.W. ~4200 kDa), is the natural ligand of the membranous G protein-coupled APJ receptor [1] which has high structural homology with the type 1 angiotensin (Ang) receptor. Both apelin and its receptor are expressed in various tissues, with a predominance in the lungs, heart, spinal cord and adipose tissue [2,3]. The peptide is hydrolyzed by the Ang-converting enzyme-related carboxypeptidase-2 (ACE2) [4].

Apelin is a multifunctional peptide, but its major actions relate to cardiovascular function, body fluid homeostasis and energy metabolism. The cardiovascular effects are well documented and fairly consistent, and include stimulation of cardiac contractility [5–9] and regulation of blood pressure [10–12]. The body fluid effects are less clear with studies suggesting both a diuretic [13,14] and an antidiuretic [15] effect. Apelin expression in adipose tissue reflects the degree of adipocyte hypertrophy [16] and likely explains the increased circulating apelin concentrations in obese humans and rodents [3]. However, apelin administration improves rather than deteriorates insulin sensitivity [17,18].

Pregnancy entails dramatic cardiovascular, body fluid and metabolic changes, both in humans and rodents. Heart rate and plasma volume increase with 40% while blood pressure decreases progressively [19]. In addition, gestational weight gain is accompanied by progressive adipocyte hypertrophy and insulin resistance [20]. Apelin may well be involved in these adaptations, but few studies have examined its role in physiological or pathological pregnancies. Based on its potential actions, apelin could be implicated in the pathophysiology of pre-eclampsia (through the regulation of blood pressure and angiogenesis), in the regulation of fetal growth (through its effects on plasma volume expansion) and in the pathophysiology of gestational diabetes mellitus (through the
regulation of glucose metabolism). To date, the peptide and its receptor have been localized in first- and third-trimester human placentas, and are markedly upregulated in preeclamptic pregnancies [21]. Fetal and neonatal plasma apelin concentrations are higher than maternal levels, but do not appear to be correlated with birth weight [22]. Finally, apelin is strongly expressed in the mammary gland of the gravid and lactating rat and is retrieved in high levels in the colostrum [23].

To gain further insight in the kinetics of apelin and as a first step toward unveiling the physiological role of apelin in pregnancy, we here examined plasma levels, relative tissue expression and clearance of apelin during rat pregnancy in a consecutive series of experiments. Since our observations pointed toward an important role for the placenta in apelin homeostasis during pregnancy, we subsequently measured apelin concentrations in pregnancies with fetoplacental reduction and studied the localization of apelin and ACE2 in the placenta.

2. Materials and methods

2.1. Animals and experimental procedures

All protocols were approved by the ethics committee for animal experimentation and the committee for safe use of radio-isotopes of the RUD Leuven. Female Sprague–Dawley rats were used, bred in-house according to Good Laboratory Practice (Le Genest Saint-Isle, France) and housed in a temperature- and humidity-controlled environment with a standard light–dark cycle. The animals were allowed to acclimatize for one week before the start of the experiments, and had free access to tap water and a standard laboratory chow (Sniff, Soest, Germany). All experiments were done between 7:00 h and 11:00 h to minimize the influence of diurnal patterns.

2.1.1. Maternal apelin plasma concentrations in pregnancy (Experiment 1)

Female rats were mated at 14 weeks of age. Successful mating was confirmed by the presence of sperm cells on a vaginal smear and defined as day 1 of pregnancy; term is day 23. Rats were randomly assigned for blood sampling on day 7, 14, or 22 of gestation (n = 7 at each time point). Another cohort of virgin non-pregnant animals was used for comparison (n = 7). The dams were anesthetized with ketamine 50 mg/kg (Parke-Davis, Zaventem, Belgium) and xylazine 10 mg/kg (Bayer, Leverkusen, Germany), both intraperitoneally. A median laparotomy was performed and blood was sampled from the descending aorta and collected into lithium-heparin tubes. The animals were euthanized by cervical elongation while anesthetized. Blood samples were centrifuged at 1200 × g and plasma was aliquoted and stored at −80 °C until further processing. This experiment also measured plasma volume and insulin-like growth factor concentrations, reported previously in Ref. [19].

2.1.2. Tissue distribution of apelin gene expression in pregnancy (Experiment 2)

Fifteen-weeks-old non-pregnant (n = 8) and time-mated day-22 pregnant (n = 8) Sprague–Dawley rats were euthanized by CO2 exposure and eight organs (brain cortex of non-pregnant rats) [24]. Apelin clearance was assessed using a radioimmunoassay (Phoenix Pharmaceuticals, Burlingame, CA) after dilution of the mixture was then purified in two steps on a Sephadex G-25 M column (PD-10, GE Healthcare, Uppsala, Sweden) and a Bio-Gel P60 column (BioRad, Hercules, CA); the week before use. The total yield of the labeling procedure was 11% resulting in a mixture containing 22 μCi/g. Fifteen-weeks-old non-pregnant (n = 8) or time-mated day-22 pregnant (n = 8) Sprague–Dawley rats were anesthetized as described above; polyethylene catheters (Degania Silicone, Degania Bet, Israel) were inserted into the jugular vein and the carotid artery. One μg of [125I]bound apelin-36 was then injected in the jugular vein, and 500-μl blood samples were drawn from the carotid artery 30, 60, 90, 120, 180, 240, 360 and 480 s after injection. The samples were centrifuged at 12000 × g and 100 μl of the supernatant was immediately read in a gamma-counter. Total protein was determined on all samples using a colorimetric assay method according to the manufacturer’s instructions (bicinchoninic acid protein assay, Fisher Bioblock, Illkirch, France) to exclude significant hemodilution.

2.1.3. Plasma apelin clearance in pregnancy (Experiment 3)

Highly purified rat apelin-(1–36) (Phoenix Pharmaceuticals, Burlingame, CA) was radiolabeled with [125I]using a modified Chloramine-T method [25]. The reaction mixture was then purified in two steps on a Sephadex G-25 M column (PD-10, GE Healthcare, Uppsala, Sweden) and a Bio-Gel P60 column (BioRad, Hercules, CA); the

2.1.4. Effect of fetoplacental mass reduction on maternal plasma apelin concentrations (Experiment 4)

Fifteen-weeks-old rats were anesthetized on day 16 of pregnancy using the medication described for experiment 1. A median laparotomy was performed and the uterus was partially exteriorized. In 8 dams, half of the gestational sacs, fetuses and placentas from each uterine horn were removed through 2 mm long incisions on the anti-mesometrial side of the uterus. The other half of the gestational sacs was left untouched. The incisions were closed with non-resorbable purse string sutures, and the animals were allowed to continue their pregnancy.

Nine other dams underwent a similar surgical procedure, including a median laparotomy and manipulation of the uterus but without removal of gestational sacs and placentas (sham procedure). On day 22, the animals were again anesthetized. Blood sampling was done and plasma apelin levels were determined as described in experiment 1. The plasma volume and insulin-like growth factor measurements in these animals have been reported previously in Ref. [19].

2.1.5. Placental apelin and angiotensin-converting enzyme 2 localization (Experiment 5)

Fifteen-weeks-old time-mated pregnant Sprague–Dawley rats were euthanized by CO2 exposure either at day 16 (n = 8) or at day 22 (n = 8) of pregnancy. In each animal, four placentas and the contiguous mesometrial triangles were rapidly dissected from the middle of the uterine horn, snap frozen in liquid nitrogen, and stored at −80 °C. Using the quantitative RT-PCR methodology described in experiment 2, relative placental and mesometrial expression of apelin and ACE2 were assessed using GAPDH as the housekeeping gene and rat ACE2 (inventoried gene expression assay Rn01462693_m1) or apelin-36 (inventoried gene expression assay Rn01462695_m1) as the target gene.

Another four placentas and mesometrial triangles were prelevated en bloc from the middle of the horns, fixed in a 4% paraformaldehyde solution and subsequently embedded in paraffin. Three μm thick sections were obtained from the placenta and mesometrial triangle, starting at the umbilical cord insertion. Sections were then deparaffinized and immunohistochemically stained for rat apelin-36 or ACE2. Endogenous peroxidase was blocked by incubation with 0.5% H2O2 in methanol for 30 min and heat retrieval was performed by a 1 h incubation at 80 °C in citrate buffer (pH 6.0). Slides were then incubated overnight with a 1:800 dilution of rabbit anti rat apelin-36 (Phoenix Pharmaceuticals, Burlingame, CA) or a 1:200 dilution of rabbit anti rat ACE2 antibodies (Abcam, Cambridge, United Kingdom).

2.2. Data analysis

We used the JMP software version 7.0 (SAS Institute, Cary, NC, USA) and Prism for windows version 5.0 (Graphpad software, San Diego, CA, USA). The normality of the data was assessed using the Kolmogorov–Smirnov normality test; all data followed a normal Gaussian distribution. Data obtained from two groups of animals were compared by two-sampled Student’s t-test when the difference in cycle threshold between target and reference gene (ΔCt) . Reference expression was calculated as the n-fold change (2−ΔΔCt) compared with a reference tissue (brain cortex of non-pregnant rats) [24]. Apelin clearance was assessed using a two-phase pharmacologic decay model. All data were expressed as means ± SEM unless specified otherwise.

3. Results

The data from experiment 1 showed that plasma apelin levels were within the non-pregnant range at day 7 and 14 of gestation; however, circulating apelin dropped by 50% in the last gestational week (Fig. 1A),
In experiment 2, we examined the relative tissue mRNA expression of apelin in both non-pregnant and day-22 pregnant rats. None of the samples had a cycle threshold over 40. We documented a very strong relative apelin mRNA expression in mammary gland tissue in the pregnant rats (200-fold stronger expression compared to non-pregnant brain tissue) (Fig. 2). We also confirmed that the lungs, adipose tissue and brain showed abundant apelin mRNA expression in comparison with brain tissue in non-pregnant and late-pregnant rats, with no difference in relative expression between both groups (all p < 0.05). The placenta expressed apelin mRNA, but only in low levels (23% that of the non-pregnant brain; z-test vs. non-pregnant 0%; p = 0.007).

In experiment 3, we documented that intravenously administered radiolabeled apelin-36 was cleared from the circulation following a biphasic elimination curve (Fig. 3). The rapid (distribution) phase was similar in non-pregnant and pregnant animals (33.1 ± 6.1 s vs. 32.4 ± 6.5 s, respectively; p = 0.84), yet the slow (elimination) phase was considerably shorter in pregnant dams than in non-pregnant animals (14.6 ± 6.1 min vs. 33.6 ± 13.7 min, respectively; p = 0.006). The measurement of plasma total protein concentration at each experimental time point did not reveal significant hemodilution due to the sampling procedure (data not shown).

Because we suspected the placenta to be responsible for the faster clearance, we measured apelin concentrations in a rat model of fetoplacental reduction (experiment 4). Litter size, fetal and placental weight of rats that underwent the fetectomy procedure were 50–60% lower than in the sham-operated group [19]. Circulating apelin concentrations on the other hand were increased by 23% in the fetectomy group compared with the sham-operated group (Fig. 1B).

Since ACE2 has been shown to hydrolyze apelin, we measured ACE2 in day-16 and day-22 placental and mesometrial tissue (experiment 5). Quantitative RT-PCR experiments showed that ACE2 was amplified in term placental tissue and that relative expression was 3.3 times higher than that of apelin in term placenta (p = 0.03; Table 1). However, ACE2 mRNA was below the detection limit in placental tissue at day 16 of gestation; nor was it detectable in the mesometrial triangle at either day 16 or day 22. Apelin-36, on the other hand, could be identified in both the mesometrial triangle and the placenta at day 16 and 22 of gestation. The mesometrial expression was 3–4 times higher than the placental expression.

4. Discussion

The current study showed that maternal apelin levels drop by 50% in the last week of normal rat gestation. This reduction is probably not the result of a decreased tissue apelin production as...
relative apelin mRNA expression in various non-reproductive tissues is virtually unaltered compared with the non-pregnant state; in addition, apelin mRNA is present in low levels both in placental and mesometrial tissue, and there is a very strong increase in apelin expression in the mammary gland in term pregnancy.

Based on the results of the exploratory experiments 1 and 2, we designed 3 other experiments to further investigate the mechanism for the lower apelin levels in maternal plasma. We suspected a changed clearance to be a potential reason for the lower plasma levels in late pregnancy, and confirmed a faster elimination of radiolabeled apelin at term in a purpose-designed experiment. As the placenta is a highly perfused metabolic tissue which grows dramatically in the last week of rat gestation, it could likely contribute to the faster elimination of apelin from plasma. To further explore this hypothesis, we measured maternal plasma apelin levels in a model of fetoplacental mass reduction, and we confirmed a 23% increase of circulating apelin levels after partial fetoplacental removal.

To gain further insight in the exact mechanism of apelin elimination during gestation, we investigated ACE2 which is currently the only enzyme shown to hydrolyze apelin [4], and we detected ACE2 mRNA expression in day-22 but not in day-16 placental tissue. We subsequently localized ACE2 immunohistochemically in the labyrinthine part of the day-22 placenta, in particular in the smooth muscle layer of the fetal arterioles. This finding, together with the increased apelin levels after fetoplacental reduction, further substantiates the role of the placenta in the clearance of maternal apelin.

Although there is a significant interspecies difference in placental anatomy, both the human and rodent placenta are hemochorial with maternal and fetal countercurrent vascular systems [26]. In rats, 2–3 centrally located maternal arterial canals prolong the spiral arteries and descend to the base of the labyrinth where they branch off into capillary spaces which extend outward and backward to the trophospongium; the capillary spaces drain into peripheral venous sinuses in the trophospongium and decidua. The fetal umbilical artery, on the other hand, branches off at the contact point with the placenta, and the arterioles run up to the labyrinth-trophospongium border. There, a dense capillary bed is formed which extends back toward the fetal side and drains into the umbilical vein. The primary function of the labyrinthine sponge is generally believed to be maternal—fetal oxygen and nutrient exchange, while the trophospongium is important for the synthesis of hormonally active substances [27]. However, the current data strongly suggest that the labyrinth also regulates the secretion and metabolism of vaso-active substances during the last week of gestation in the rat.

In a previous study in rat pregnancy, ACE2 mRNA expression was found to be higher in the placenta than in the uterine wall or the kidneys, with the total estimated placental ACE2 activity amounting to ~30% of the renal activity [28]. Although we did not investigate ACE2 activity in the current study and increased ACE2 tissue levels do not necessarily mean an augmentation in total enzyme activity, previous work from Levy et al. [28] has shown that ACE2 activity doubles during gestation, mainly due to the contribution of the placenta. Neves et al. [29] also reported faint ACE2 immunostaining in the labyrinth of the rat day 19-placenta; however, no cellular localization was provided. They also found positive staining in the mesometrial triangle, which we did not confirm in the present study [29].

The present data cannot exclude the possibility that ACE2 activity in extraplacental tissues contributes to the reduction in circulating apelin in late gestation. Indeed, ACE2 mRNA expression has also been identified in adipose tissue, the gastro-intestinal tract, the renal system, the heart and the lungs [37]. Nevertheless, the data from experiment 4 in which fetoplacental mass reduction resulted in higher maternal plasma apelin concentrations, underscores the importance of placental ACE2 in the kinetics of apelin.

Apart from the catabolic hydrolysis of apelin, ACE2 inactivates angiotensin I (AngI) to Ang(1–9) and hydrolyzes AngII to Ang(1–7) with a 400-fold higher catalytic efficiency [4]. Ang(1–7) binds to the Mas-receptor and causes vasodilation, thereby counter-balancing the vasoconstrictory effects of AngII [30]. Interestingly, apelin and AngII share more than their metabolic pathway. APJ and the angiotensin receptor type 1 show ~30% structural homology, and their postreceptor signaling pathways show high similarity in the heart and in smooth muscle [31]. Moreover, apelin and AngII trigger opposite (patho)physiological processes and show opposite dynamics with apelin levels increasing when AngII decreases [7,31–34]. The current study further substantiated this finding in physiologic pregnancy showing decreased apelin levels at term, which appears to coincide with an increase in AngII and Ang(1–7) [35]. Pre-eclampsia on the other hand, which is accompanied by a relative decrease in Ang(1–7) [36], is associated with an upregulation of apelin and APJ immunoreactivity in the human placenta [21].

The rat placenta and mesometrial triangle express apelin mRNA at a low level (23% of level in non-pregnant brain) and we documented a low to moderate immunostaining of smooth muscle cells of fetal arterioles in the labyrinth. The loco-regional effects of apelin are still unknown. Interestingly however, apelin administration was found to produce vasodilatory effects in the rat glomerulus [14]. It is therefore possible that placental apelin regulates the vascular tone of fetal arterioles, and might thereby modulate the transplacental nutrient transfer. Further studies are mandatory.

The current data may be relevant for normal and abnormal human pregnancy. To our knowledge, no longitudinal data are available for apelin in the human placenta.

Table 1

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<th>Table 1: Relative expression of apelin-36 and ACE2 in the placenta and mesometrial triangle.</th>
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<td>Apelin-36</td>
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<td><strong>Placenta</strong></td>
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<td><strong>Mesometrial triangle</strong></td>
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<td>Day 16</td>
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Legend: Day 22 apelin-36 expression was set as the reference (relative expression = 1). NA: no significant amplification.
available on plasma apelin levels throughout human pregnancy. One recent study reported no difference between 101 normal gravidas at 27–30 weeks and 16 gravidas at 37–40 weeks [38], but non-pregnant or early pregnancy values were not available, precluding definitive conclusions. Apelin and APJ have been identified in cytotrophoblasts and syncytiotrophoblast, stroma and endothelial cells of the human placenta; the apelin but not APJ immunoreactivity decreased from the first to the third trimester in normal pregnancies [21]. In addition, ACE2 is expressed in term human placentas at the level of the trophoblasts, the endothelium and vascular smooth muscle of the primary villi [39]. On the basis of these data, we would expect human apelin levels to follow the same trend as in rats.

This study did not examine the physiologic effects of apelin during rat pregnancy. However, we have previously reported data on plasma volume for the animals of experiments 1 and 4 [19]. Further examining these in respect to apelin levels, we found a negative correlation between plasma volume and apelin both in physiologic pregnancy (Experiment 1; \( r = -0.64; p = 0.0006 \)) and in the animals undergoing the fetectomy and sham procedure (Experiment 4; \( r = -0.56; p = 0.03 \); unpublished data). This negative correlation might be explained by the observation that both plasma volume expansion [19] and the drop in circulating apelin during the third week of rat pregnancy appear to be regulated by the functional placental mass.

Clearly, further studies are needed to document the role of the placenta, and of apelin and ACE2 in particular, on cardiovascular function, body fluid homeostasis and glucose metabolism during pregnancy. Indeed, based on the currently known (patho)physiologic effects of apelin, alterations in apelin levels in gestation could be involved in angiogenesis [40], in the regulation of blood pressure, plasma volume expansion and cardiac function which would all be of importance in the study of pre-eclampsia and fetal growth restriction. Moreover, disturbances in apelin physiology could play a role in glucose metabolism.

In conclusion, we showed that maternal plasma apelin levels decrease in the last week of rat gestation likely due to increased elimination by the fetoplacental unit. Given the presence of ACE2 in the smooth muscle cells surrounding the fetal arterioles in the labyrinth, we speculate that the labyrinthine portion of the placenta is responsible for the increased apelin metabolism in gestation.

**Fig. 4.** Parallel sections through a fetal arteriole running up the labyrinth of a day-22 rat placenta stained for (A) apelin-36, (B) ACE2 and (C) actin, demonstrating the presence of these proteins in the perivascular smooth muscle. (D) Apelin-36 staining of the mesometrial triangle at term demonstrating faint apelin staining in the cytoplasm of stromal cells. (E) Negative control of labyrinthine section demonstrating a fetal artery without any staining at the level of the perivascular smooth muscle. Staining is only present in the red blood cells.
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Disclosures

All authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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