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## ASSESSMENT OF ANGIOTENSIN I METABOLISM IN THE HUMAN PLACENTA USING AN LC/MS METHOD

**Abstract:** The local renin-angiotensin system (RAS) in the placenta plays a very important role in placental development. It is well known that during normal pregnancy most of the circulating and local RAS components are over-expressed and any disruption of this new balance may cause pregnancy complications. The aim of this study was to assess the metabolism of Ang I in placentas from normal pregnancy, in an *ex vivo* model, using an LC/MS method. The obtained results suggest that placental tissue is able to produce many angiotensin peptides but the main metabolite is Ang-(1–7).

**Key words:** Angiotensin, placenta, mass spectrometry, metabolism.

### INTRODUCTION

The renin-angiotensin system (RAS) (the main pathways of the production of different angiotensins are presented in Fig. 1) plays a significant role in regulating arterial blood pressure and the water-electrolyte balance. During pregnancy, a number of RAS components are overexpressed, both in the blood and in tissues [1]. Due to the increase in extrarenal renin production, its plasma levels rise, and the increase in the concentration of circulating oestrogens results in higher production of angiotensinogen in the liver. Angiotensin II (Ang II) also reaches higher plasma concentrations, though the angiotensin-converting enzyme (ACE) is a RAS component whose plasma level decreases in pregnancy [2, 3]. The presence of an active, local RAS in many tissues, including the placenta, has been proven. The placental RAS can be involved both physiologically and pathophysiologically in the processes occurring during pregnancy. It is well-known that Ang II takes part in the regulation of vascular resistance and blood flow in pregnancy — low concentrations of Ang II increase uteroplacental blood flow and high concentrations decrease it [4]. In the mother's cardiovascular system, in normal pregnancy, the ability to generate angiotensin peptides rises, but blood vessels become

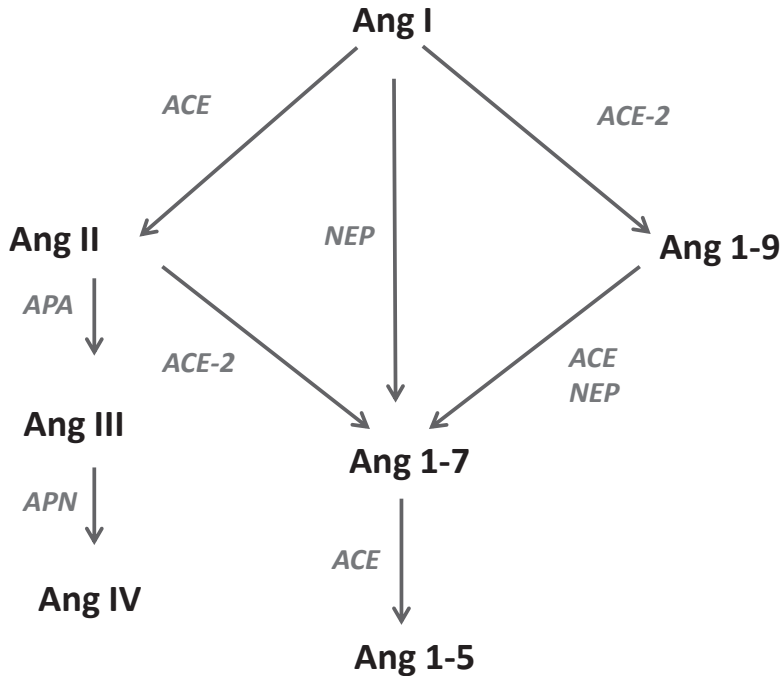


Fig. 1. The main pathways of Angiotensin I metabolism (APA — aminopeptidase A; APN — aminopaptidase N; NEP — neutral endopeptidase; ACE — angiotensin converting enzyme; ACE2 — angiotensin converting enzyme type 2).

less sensitive to Ang II, which results in resistance to its pressor activity. To obtain the same vasomotor response as in non-pregnant women, pregnant ones require intravenous doses of Ang II that are twice as high [1, 5].

In studies carried out over the last decade, there were suggestions that a recently described heptapeptide — angiotensin-(1-7) (Ang-(1-7)) — may play an important role in the regulation of arterial blood pressure during pregnancy [6-9]. Ang-(1-7) acts as an antagonist of Ang II — it exhibits vasodilating properties by releasing NO, kinins and prostacyclin. Contrary to Ang II, Ang-(1-7) decreases arterial blood pressure and inhibits angiogenesis. It has been shown that serum concentrations of Ang-(1-7) in patients in normal pregnancy reach much higher levels in comparison with pregnant women with pre-eclampsia. It is probable that changes in Ang-(1-7) levels, and particularly in the Ang-(1-7)/Ang II ratio, play a significant role in the pathogenesis of pre-eclampsia [8].

The studies conducted so far have shown the presence of individual RAS components in the placenta — angiotensinogen, Ang I and Ang II as well as enzymes degrading each peptide and Ang II receptors. The studies were conducted on tissues originating from human placentas, cell cultures and animal models, using immunoenzymatic, biochemical and molecular methods. There is little information

about the production of Ang I metabolites other than Ang II and Ang-(1-7), which can also exhibit biological activity.

The aim of this study was to examine the metabolism of angiotensin I, as a precursor of other angiotensins, in sections of placentas originating from normal pregnancies. In the study, we used an *ex vivo* model of angiotensin peptide generation, supplemented by the measurement of concentrations of each angiotensin using the high performance liquid chromatography mass spectrometry (LC/MS) method [10, 11].

## MATERIAL AND METHODS

The study was carried out at the Department of Obstetrics and Perinatology and the Chair of Pharmacology of Collegium Medicum at the Jagiellonian University. In the study, we used sections of placentas coming from patients ( $n = 5$ ) in normal pregnancy, without any concomitant chronic diseases or abnormalities found in the foetus, scheduled for elective caesarean sections (abnormal foetal presentation, foetopelvic disproportion). Tissues for examination were collected immediately after the caesarean sections ( $1 \times 1$  cm placental specimens from the area of the umbilical cord), washed with a refrigerated 0.9% NaCl solution, closed in a sterile container with refrigerated 0.9% NaCl and transported to the Chair of Pharmacology, where assays were performed. The time between the collection and the delivery of the material did not exceed 40 minutes in any of the cases. The research procedures were covered by the consent of the Bioethics Committee No. KBET/345/B/2012.

## TISSUE INCUBATION

The delivered sections of placentas were washed with normal saline at room temperature, then cleaned off any remaining thrombus and divided into smaller pieces. The cleaned sections of placentas were incubated in Krebs-Henseleit buffer at the temperature of 37°C for 15 minutes in the presence of Ang I (the final concentration: 3  $\mu$ M). The incubation of control samples was carried out with the same method (only buffer or buffer with Ang I, without tissue).

After incubation, the supernatant was secured for LC/MS analyses, while tissue sections were dried at the temperature of 60°C and weighed.

## PREPARATION OF SAMPLES FOR LC/MS ANALYSES

The supernatants from the incubation were purified and concentrated using Ultra-Micro Spin C-18 columns (Harvard Apparatus, USA). The columns were conditioned (300  $\mu$ l of 0.1% TFA in 80% acetonitrile, 300  $\mu$ l of 0.1% TFA) before

placing samples in them. 200  $\mu$ l of each sample was acidified with formic acid to obtain pH <3, and then placed in the column and centrifuged (2 min, 1000  $\times$  g). Subsequently, the columns were washed with 300  $\mu$ l of 0.1% TFA and angiotensin peptides were eluted with 300  $\mu$ l of 0.1% TFA in 40% acetonitrile (2 min., 1000  $\times$  g). The eluates were lyophilised overnight; then their dry remains were treated with 50  $\mu$ l of 0.1% formic acid and subjected to LC/MS analysis. Samples for calibration curves, containing mixtures of standards of the assayed angiotensins, and control samples were prepared in the same way.

### ANALYSIS OF THE PRODUCTS OF ANG I METABOLISM USING THE LC/MS METHOD

The chromatographic separation of angiotensin peptides was carried out with the use of a HPLC reversed-phase system (Ultimate 3000, Dionex USA), in an Acclaim PepMap 100 column (LC Packings, CA, USA), RP C18 (150 mm  $\times$  300  $\mu$ m ID, 5  $\mu$ m particle size).

The gradient flow of mobile phases was used: A — 4 mM ammonium formate buffer with 4 mM formic acid in 5% acetonitrile; B — 4 mM ammonium formate buffer with 4 mM formic acid in 95% acetonitrile. The flow rate of the mobile phases was 5  $\mu$ l/min.

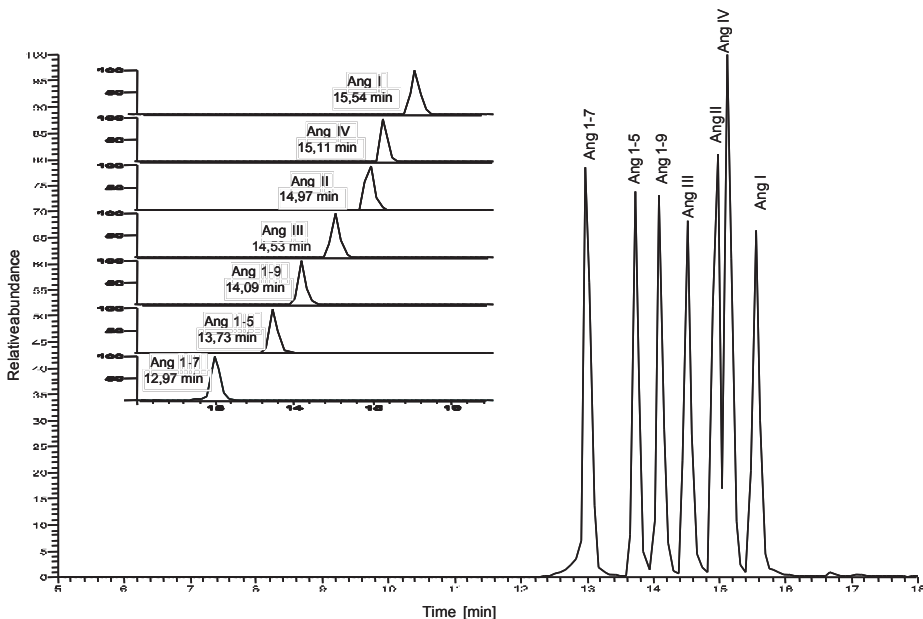


Fig. 2. TIC chromatogram of angiotensins peptides standards. The insert presents extracted ions with masses corresponding to each peptide.

The detection of the examined substances was carried out using an LCQ mass spectrometer (Finnigan Mat, San Jose, USA) with an ESI ion source (liquid-junction). The working parameters of the ESI-MS were as follows: electrospray needle voltage 2.5 kV; capillary voltage -5V; capillary temperature 200°C, positive ion mode. The SIM (selected ion monitoring) option was used in ion acquisition, established during previous studies [10–13]. The obtained data was analysed with Xcalibur v.2.0 software. The separation of the standards of individual angiotensins is presented in Fig. 2.

The concentrations of each angiotensin peptide were determined on the basis of calibration curves, determined using linear regression of the area under the peak in the peptide concentration function. The obtained values were converted to mg of dry tissue.

## REAGENTS

The following reference substances and reagents were used during the experiments: angiotensin peptide standards: Ang I, II, III, IV, 1–9, 1–7 and 1–5 were produced by Bachem, Switzerland; ammonium acetate, trifluoroacetic acid (TFA) and formic acid were manufactured by Fluka, Switzerland; acetonitrile was manufactured by Merck, USA.

The solutions of the reference substances, buffers and mobile phases for chromatographic separation were prepared with water obtained from a Simplicity Milliopore system (Merck Millipore, USA).

## STATISTICAL ANALYSIS

The results are presented as mean values  $\pm$  standard deviation. The significance of differences between the groups was tested with the use of one-way analysis of variance (ANOVA) as well as Scheffé's *post hoc* test. A value of  $p < 0.05$  was accepted as statistically significant.

## RESULTS

The sections of placentas exhibited an ability to convert Ang I to other metabolites. In the analysed samples, the presence of all the assayed metabolites of angiotensin I was shown (Fig. 3). *Ex vivo*, the main product of Ang I degradation turned out to be angiotensin-(1–7). Its production was  $57.27 \pm 9.14$  ng/mg. The next two main Ang I metabolites were Ang II ( $5.31 \pm 1.74$  ng/mg) and Ang-(1–5) ( $3.97 \pm 0.51$  ng/mg). The remaining assayed metabolites — Ang-(19), Ang III and Ang IV — were produced in much lower quantities ( $<1$  ng/mg).

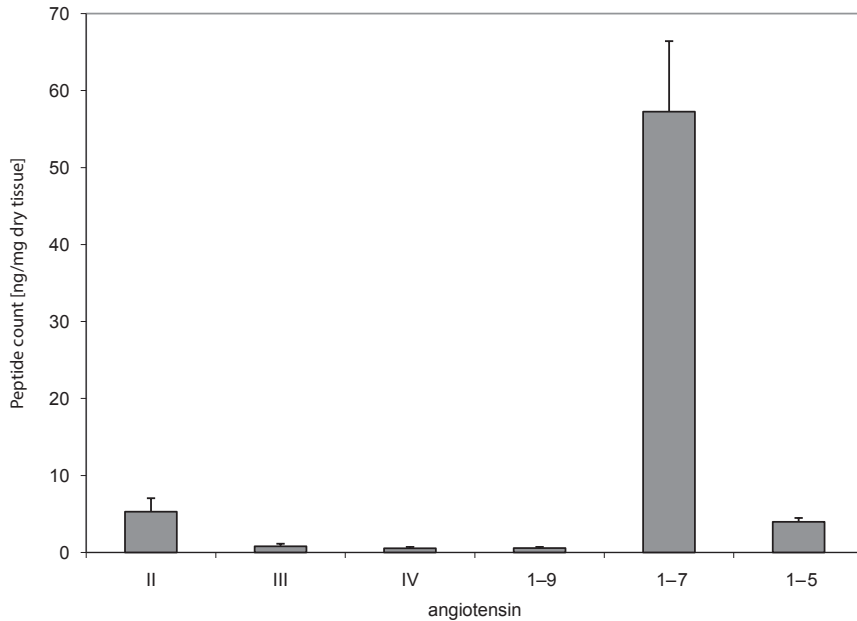


Fig. 3. The levels of angiotensin peptides produced during incubation of placenta sections in the presence of Ang I.

## DISCUSSION

In this study, we showed that placental tissues coming from normal pregnancy are capable of degrading (converting) angiotensin I. The resulting Ang I degradation products require the presence of a number of enzymes, e.g. ACE, ACE2, NEP, chymases or aminopeptidases. During our study, we found that Ang-(1-7) is the main metabolite in placental tissues in normal pregnancy, which is consistent with other reports, which also showed the dominant production of Ang-(1-7) not only in the tissue RAS, but also in the plasma RAS [1, 9, 14].

In the classic approach, the renin-angiotensin system (RAS) is an endocrine system, in which the main, biologically active octapeptide — angiotensin II — is produced in plasma, as a result of the activity of angiotensin convertase (ACE), from a decapeptide, angiotensin I, separated from angiotensinogen (produced by the liver) by renin, released, in turn, by the ischaemic kidneys [15] (Fig. 1). In this approach, the biological effects of RAS stimulation depend mostly on the systemic activity of Ang II. In recent years, this traditional view of the RAS has changed considerably. It turned out that Ang I and Ang II can be converted by peptidases other than ACE (e.g. neutral endopeptidase — NEP — or the recently

discovered ACE2 isoform), promoting the occurrence of “non-typical” products [e.g. angiotensin-(1-7) or angiotensin-(1-9)], which often have specific receptors and perform important biological functions. Also, the presence of most RAS components in a number of tissues (the tissue RAS) has been found and attention has been drawn to the important, paracrine activity of different angiotensins (often those “non-typical” ones), occurring locally, within individual organs. The most research has been devoted to the activity of the “local” RAS in the kidneys, heart, brain and the reproductive system. As it turns out, in all these areas, significant functions of organs are regulated by the activity of different angiotensins balancing each other; their concentrations are determined by the tissue-specific activity profiles of each converting enzyme. The balance between Ang II and Ang-(1-7) is the clearest [16, 17].

The research into the RAS in the physiology and pathophysiology of pregnancy conducted so far focuses mostly on the role of Ang II and Ang-(1-7). It has been shown that the expression of most RAS components changes as pregnancy progresses, both in the circulation and tissues. It is caused by hormonal changes occurring in the body of a woman during pregnancy. Oestrogens in uncomplicated pregnancy cause a shift in the production of angiotensin — the generation of Ang-(1-7) is increased and Ang II is reduced [18]. The observed decreased sensitivity to Ang II in normal pregnancies may be associated with the different structure of the type 1 angiotensin receptor (AT1), occurring then in the form of a monomer, in contrast with abnormal pregnancies, in which sensitivity to the activity of Ang II occurs, and the AT1 receptor has a heterodimeric structure [14, 19]. A disruption of the local RAS balance can result in the development of pregnancy complications, such as pre-eclampsia. In pregnancies complicated by pre-eclampsia, a lower expression of RAS components has been observed: lower concentrations of renin, Ang I and Ang II in comparison with normal pregnancies [20]. In addition, it has been shown that, in patients with pre-eclampsia, the sensitivity to Ang II is increased, which manifests itself as increased pressor response to the activity of Ang II [1, 14].

In recent years, researchers have been stressing the important role of Ang-(1-7) in the normal functioning of the placenta [8, 14]. In a study of plasma concentrations of Ang I, Ang II and Ang-(1-7) in pregnant patients (normal and complicated pregnancies) and non-pregnant ones, comparing Ang-(1-7)/Ang II, Ang-(1-7)/Ang I and Ang II/Ang I concentration ratios, a significant decrease in the value of all ratios in the non-pregnant women vs. women in normal pregnancies group has been shown, while the non-pregnant vs. pregnant with pre-eclampsia group only the Ang-(1-7)/Ang II ratio was significantly reduced (that is, there was a decrease in Ang-(1-7)). A negative correlation between the level of Ang-(1-7) and arterial blood pressure during pregnancy has been proven as well [8].

In this study, we showed that the main product of Ang I degradation in placental tissue originating from a normal pregnancy is Ang-(1-7). The placenta also

exhibits the ability to form other examined metabolites of Ang I, though they are produced in much smaller amounts. The *ex vivo* model used in the presented study and the LC/MS method, successfully used previously in research into Ang I, Ang-(1–12) and angiotensinogen conversions in different tissues (the aorta, heart, kidneys, adipose tissue) [10–13], allowed a comprehensive evaluation of angiotensin I conversion in placental tissues. Further research, extended to cover tissues coming from placentas from complicated pregnancies, using the presented method, may allow us to estimate changes in the production of each metabolite in different pathological states in the course of pregnancy, and thus shed new light on the impact of disruption in the placental metabolism of angiotensins on the development of these abnormalities.

### SUMMARY

The aim of this study was to investigate the metabolism of angiotensin I as a precursor of other angiotensins, in sections of placentas originating from normal pregnancies. An *ex vivo* model of angiotensins peptides generation developed earlier was applied. The measurement of individual angiotensin concentrations was performed with the high-performance liquid chromatography coupled with mass spectrometry (LC/MS) method. We proved that the main product of Ang I metabolism in placental tissue derived from physiological pregnancy is Ang-(1–7). The placenta also exhibits the ability to generate other Ang I metabolites, although they are produced in much smaller quantities. The *ex vivo* model allowed a comprehensive assessment of angiotensin I changes in healthy placental tissues. Further studies, extended to cover tissues from complicated pregnancies, may allow us to evaluate any changes in the production of various metabolites during pathological conditions in pregnancy, and thus shed light on some problems associated with the placental metabolism of angiotensins in the development of these abnormalities.

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