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ANGIOTENSINOGEN METABOLISM IN RAT AORTA:
ROBUST FORMATION OF PROANGIOTENSIN-12

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Renin-angiotensin system (RAS) plays important role in the regulation of vessel wall homeostasis. Proangiotensin-12 (proAng-12, Ang-(1-12)) is a newly characterized metabolite of angiotensinogen, formed in array of organs of rats, which may serve as an alternate substrate for local angiotensin production, by-passing the traditional renin-dependent conversion of angiotensinogen to angiotensin I. In this work using LC/MS method we identified proAng-12 as a main product of angiotensinogen metabolism ex vivo, in organ-bath of rat aortic tissue. In this setting, proAng-12 appeared to be not only prevalent metabolite of angiotensinogen, but also served as a substrate for generation of Ang I and subsequently, Ang II. The functional significance of this surprising finding requires further investigation.

Key words: angiotensin, angiotensin converting enzyme (ACE), proangiotensin-12, renin

INTRODUCTION

Our view of renin-angiotensin system (RAS) has experienced remarkable change over the past decades. The new enzymes (e.g. angiotensin converting enzyme 2, (ACE2)) and products (e.g. Ang-(1-7)) as well as their local formation and autocrine and/or paracrine action have been described (1, 2). Recently, Nagata et al. using EIA method reported the presence of new angiotensinogen metabolite - proangiotensin-12 (proAng-12, Ang-(1-12)) - in several rat tissues; it has been detected mainly in small intestine, and to less extent in spleen, kidneys, liver and heart (3). For at least two reasons this finding may shed a new light on RAS function: (i) in contrast to the classical pathway of Ang I generation, the formation of proAng-12 from angiotensinogen has been shown to be independent on renin activity (4, 5) (ii) moreover, at least in the heart, proAng-12 has been shown to be directly converted to Ang II (by-passing classical ACE-dependent pathway) (Fig. 1) (5-7). Thus, formation of proAng-12 could represent renin- and ACE-independent component of tissue Ang II production, which is not “druggable” by renin- and ACE-inhibitors (1, 8).

It is widely accepted that RAS plays crucial role in the regulation of vessel wall homeostasis and efficient targeting of Ang II formation in vessel wall is of great importance for pharmacotherapy of chronic cardiovascular diseases (9, 10). ProAng-12 may serve as an alternate substrate for angiotensin production, however neither the extent nor the enzyme(s) responsible for its generation in vascular tissue have been determined.

Mass spectrometry coupled with liquid chromatography (LC/MS) has proved to be reliable method of detection and quantification of peptide compounds in biological samples, devoid of EIA problems with specificity (11). In this work we applied LC/MS method to study the formation of proAng-12 from angiotensinogen ex vivo, in organ-bath of rat aorta.

MATERIALS AND METHODS

Isolation and treatment of rat tissues

Male Wistar rats at 7 months of age and 340-410 g of weight were administered fraxiparine (2850 IU, i.p.) and anaesthetized with 50 mg of thiopentone (50 mg/ml, i.p.). Fragments of aorta were excised through abdominal incision, washed with cold, standard Krebs-Henseleit solution and cleaned of thrombi and tissue remnants. Blood vessels were cut into a suitable number of rings and opened flat. Tissue incubation was done as described previously (12, 13). Briefly, tissue fragments were incubated for 30 minutes at 37°C in Eppendorf tubes in 550 µl of Krebs-Henseleit solution and continuously bubbled with 95%O2/5%CO2.

Sample of 50 µl of buffer was removed to provide information on background production of angiotensin metabolites. Then, Ang-(1-12) or Ang-(1-14) were added to a final concentration of 1 µM. Samples of 50 µl of buffer were removed after another 15 min of incubation. Each sample was promptly frozen at -70°C until further analysis. Tissue pieces were dried overnight at 60°C to allow estimation of angiotensin metabolites’ production per mg of dry tissue. All procedures were approved by an Ethical Committee of the Jagiellonian University, School of Medicine.

Sample preparation

Samples were purified and concentrated using Ultra-Micro Spin C-18 column (Harvard Apparatus, USA). Briefly, 200 µl of
sample were applied on columns and centrifuged (2 min, 1000xg). Then columns were washed with 300 µl of 0.1% TFA by centrifugation as above. Finally angiotensin peptides were eluted by centrifugation with 300 µl of 0.1% TFA in 40% acetonitrile. Then the samples were lyophilized overnight, and dry remainings were reconstituted in 0.1% TFA for LC-MS analysis. Samples for calibration curves of each examined peptides (mixture of standards) were prepared in the same mode as above.

**Measurement of angiotensin peptides concentration by LC-MS method**

Separation of angiotensin peptides was performed on a reversed-phase, high performance liquid chromatography (HPLC) system Ultimate 3000 (Dionex, USA) as described previously (12, 13). The column used for separation was Acclaim PepMap 100 C18 column (150 mm x 300 µm ID, 5 µm particle size) with a guard column (5 mm x 1 mm; 5 µm particle size) working at a flow rate of 5 µl /min (Dionex, USA).

Mass spectrometric detection was performed using a LCQ ion-trap mass spectrometer (Finnigan, San Jose, USA), equipped liquid-junction ESI ion source (ion spray voltage 2,5 kV; capillary voltage -5 V, capillary temperature 200°C). For angiotensin peptides detection, selected ion monitoring (SIM) mode was used, set at 450,32 Da for Ang-(1-7); 466,38 Da for Ang III; 523,95 Da for Ang II; 592,39 Da for Ang-(1-9); 648,95 Da for Ang I; 665,27 Da for Ang-(1-5); 775,27 Da for Ang IV; 787,11 Da for Ang-(1-12) and 912,14 Da for Ang-(1-14).

Acquired data were analyzed by Xcalibur Software, version 2.0. Concentrations of angiotensin peptides were calculated using the standard calibration curves, constructed by linear regression analysis plotting of peak area versus peptide concentration. Calibration curves were prepared for each examined peptide at a concentration range of 2.5-250 ng/ml.

**Chemicals**

Rat angiotensinogen fragment: H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Tyr-Ser-OH (angiotensinogen 1-14; Ang-(1-14)), rat angiotensin 1-12 (Ang-(1-12)), angiotensins: I (Ang I), III (Ang III), IV (Ang IV) and angiotensin fragments 1-9 (Ang-(1-9)) and 1-5 (Ang-(1-5)) were purchased from Bachem (USA). Angiotensin II (Ang II) and angiotensin fragment 1-7 (Ang-(1-7)) were purchased from Sigma Chemicals (USA). Formic acid (99%), trifluoroacetic acid (TFA) and ammonium formate were purchased from Fluka (USA). Acetonitrile (J.T. Baker, USA), and water (Rathburn, Scotland) were HPLC grade.

**Statistics**

Concentrations of angiotensins were expressed as in pmol/mg dry tissue. All values in the figures and text are expressed as mean±S.E. of n observations.

**RESULTS**

Incubation of 1-14 fragment of angiotensinogen for 15 min with rat aorta resulted in production of high amount of Ang-(1-12) (Fig. 2A). Importantly, the production of Ang-(1-12) appeared to be almost 7-fold higher than the formation of Ang I. Metabolism of angiotensinogen in rat aorta resulted also in formation of significant amounts of Ang II, Ang-(1-7) and Ang-(1-9) (Fig. 2A).

*Fig. 1.* Main pathways of angiotensinogen metabolism. ACE - angiotensin converting enzyme, ACE2 - angiotensin converting enzyme type 2, APA-aminopeptidase A, APB - aminopeptidase B, NEP - neutral endopeptidase.
In rat aorta, Ang-(1-12) was mainly metabolized to Ang I (Fig. 2B). Metabolism of Ang-(1-12) resulted also in significant amounts of Ang II, Ang-(1-7) and Ang-(1-9) (Fig. 2B).

**DISCUSSION**

Incubation of rat aorta with angiotensinogen resulted in a robust formation of whole bunch of angiotensin peptides. Surprisingly, in this setting we identified proAng-12 as a main product of angiotensinogen metabolism. Importantly, proAng-12 was not only prevalent metabolite of angiotensinogen, but also served as a substrate for generation of Ang I and subsequently, Ang II. The high ability of aortic tissue to proAng-12 formation is surprising, taking into granted previous reports showing low levels of endogenous proAng-12 in aorta, determined by radioimmunoassay (3). This discrepancy may result from the short half-life of proAng-12 in tissues and/or differences between LC/MS and EIA methods of detection. It should be noted however, that our assay reflects mainly ability of tissue to form proAng-12 in the presence of high amount of substrate, so it may well be that the tissue/blood levels of angiotensinogen constitute main limiting factor for the local formation of proAng-12 in vivo. According to the data of Campbell *et al.* the levels of...
angiotensinogen within aortic wall are by 70% lower than in the liver (14). However, the concentrations of circulating and aortic tissue angiotensinogen could be significantly increased by action of corticosteroids, thyroid hormones and Ang II itself as well as were shown to be elevated during pregnancy, severe inflammation, increased salt intake and chronic kidney disease (15-20). We are tempted to speculate that in these clinical situations the endogenous proAng-12 may start to play important role in tissue formation of Ang I and Ang II.

Systemic administration of proAng-12 is associated with hypertensive responses (5). However, besides conversion to Ang I (probably by ACE) and Ang II (by chymase) proAng-12 could be also directly metabolized by NEP to vasodilatatory and anti-atherosclerotic Ang-(1-7) (1, 21). Apparently, the fate of proAng-12 seems to be determined by pattern of tissue activities of particular proteases.

The functional significance proAng-12 is not fully elucidated. Trask et al. suggested that proAng-12 may serve as a versatile, renin-independent "quick release" substrate for immediate production of RAS components in tissues, instead of slower cell making of the large angiotensinogen molecules (c.a. 500 AA) (5). In support of such notion, Oparil et al. recently showed that in patients treated with maximal doses of the renin inhibitor aliskiren addition of angiotensin II type 1 receptor antagonist valsartan resulted in further reduction of blood pressure, which was quite unexpected finding if renin is the sole liberator of angiotensin peptides (22).

Although preliminary, our study underline high ability of aortic tissue to formation of proAng-12 from angiotensinogen. It was not aimed to investigate either the biochemical pathways or the functional significance of proAng-12 in regulation of vessel wall homeostasis. These and other questions, like the interference of proAng-12 with actions of RAS-targeted drugs, require further investigation.

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Conflict of interests: None declared.

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