PERINDOPRILAT CHANGES ANG (1-9) PRODUCTION IN RENAL ARTERIES ISOLATED FROM YOUNG SPONTANEOUSLY HYPERTENSIVE RATS AFTER ANG I INCUBATION

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SUMMARY:

We used mass spectrometry to quantitate production of angiotensinogen metabolites in renal artery of 3 and 7 months old Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR). Tissue fragments were incubated for 15 minutes in oxygenated buffer, with added Angiotensin I. Concentrations of Angiotensins I (ANG I), II (ANG II), III (ANG III), IV (ANG IV), Angiotensin (1-9) [ANG (1-9)], Angiotensin (1-7) [ANG (1-7)], and Angiotensin (1-5) [ANG (1-5)], excreted into the buffer during experiment, were measured using liquid chromatography- mass spectrometry (LC/MS) and expressed per mg of dry tissue.

Effects of pretreatment with 10 μM perindoprilat on the production of ANG I metabolites were quantitated. Background production of any of ANG I metabolites differed neither between WKY and SHR rats nor between 3 and 7 months old rats.

Perindoprilat pretreatment of renal arteries resulted, as expected, in decrease of ANG II production. However, renal arteries of 7-month-old SHR rats were resistant to ACE inhibitor and did not change ANG II production in response to perindoprilat. In renal arteries, taken from 3-month-old rats, pretreated with perindoprilat, incubation with ANG I, resulted in the level of ANG (1-9) significantly higher in SHR than WKY rats.

Our conclusion is that in SHR rats, sensitivity of renal artery ACE to perindoprilat inhibition changes with age.

1. INTRODUCTION

Angiotensinogen metabolites play a prominent role in vasculature. For the long time, the most extensively studied pathway of metabolism of angiotensin I (ANG I) was formation of angiotensin II (ANG II), III (ANG III), and IV (ANG IV) through action of angiotensin converting enzyme (ACE) and aminopeptidases. Recently, cleavage of amino acids at the carboxy terminus of ANG I molecule, which leads to formation of angiotensins (1-9) (ANG (1-9)), (1-7) (ANG (1-7)), and (1-5) (ANG (1-5)) became also a subject of intense research (Vickers *et al.* 2002, Ferreira *et al.* 2012, Santos *et al.* 2013). Cleavage of carboxy terminal amino acid from ANG I to form ANG (1-9), mediated by angiotensin converting enzyme 2 (ACE 2), was discovered in the year 2000 (Donoghue *et al.* 2000, Tipnis *et al.* 2000). Other reactions in this pathway are mediated by cathepsins and neutral endopeptidases (Uehara *et al.* 2013, Jackman *et al.* 2002). Interest in this alternative pathway of ANG I metabolism is substantiated by the vasodilatatory and antiproliferative, i.e., cardioprotective effects of ANG (1-7), which are contrasted with vasoconstrictive and proliferative effects of ANG II (Chappell 2007, Iyer *et al.* 1998). Overproduction of ANG II plays an important role in development of hypertension and inhibitors of ACE are the cornerstone of antihypertensive therapy (Luque *et al.* 1996, Ferrario *et al.* 2005, Ferrario 2010). However, no drugs, targeting carboxy terminus cleaved angiotensins were developed for clinical use, so far.

Local renal RAS plays an important role in the development of kidney failure in hypertension (Obata *et al.* 2000, Ma *et al.* 2014, Zhuo *et al.* 2013) or in diabetic nephropathy (Soler *et al.* 2008, Ribeiro-Oliveira *et al.* 2008). Treatment with inhibitors of angiotensin converting enzyme (ACE-I) or with blockers of ANG II receptor (AT_1) is

still a cornerstone of therapy in patients with hypertension and diabetic kidney diseases (Ferreira *et al.* 2012, Luque *et al.* 1996, Ferrario *et al.* 2005).

Tikellis *et al.* described developmental changes in ACE and ACE2 expression and activity in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls (Tikellis *et al.*, 2006). Interestingly, ACE but not ACE2 activity was lower in SHR rats compared to WKY controls. The aim of our study was to determine whether sensitivity of renal arteries to ACE inhibition changes with age and how this impacts ANG I metabolism in SHR and WKY rats.

Inbred strain of SHR rat is a well-established model of genetically determined hypertension. We incubated in water bath fragments of renal artery, isolated from SHR and WKY rats at the age of 3 months (when hypertension reaches *plateau*, Tikellis *et al.* 2006) and at 7 months (before the onset of kidney damage, Braun *et al.* 2013) to quantitate, by LC/MS method, production of ANG I metabolites. The effects of perindoprilat (an ACE-I) on angiotensin metabolism were also studied.

2. MATERIALS AND METHODS

2.1.Animals:

Male WKY and SHR rats, 3-month-old (3M, n=9 for each strain), 330-400g and 310-360g of weight, respectively and 7-month-old (7M, n=10 for each strain), 340-410g and 400-440g of weight, respectively, were administered fraxiparine (2850 IU, i.p.) and anesthetized with 50 mg of thiopentone (50 mg/ml, i.p.). Fragments of renal artery were excised through abdominal incision. All experimental studies using animals were approved by an Ethical Committee at the Jagiellonian University (No 12/2006).

2.2.Preparation of tissue fragments and "organ-bath" procedure

Tissue fragments were 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 rectangular rosettes of about 2 mm^2 each. Tissue incubation was performed as described previously (Bujak-Giżycka *et al.* 2007) in our earlier established and optimized protocol. Briefly, tissue fragments were incubated in triplicates, each in 400 μl of freshly prepared, 95% oxygen and 5% CO_2 bubbled Krebs buffer (at 37°C) with or without an ACE-I - perindoprilat at final concentration of 10 μM. Before addition of any exogenous substance, sample of 50 μl of buffer was removed to provide information on background levels of ANG I metabolites. After 5 minutes of incubation with perindoprilat or buffer, ANG I was added to a final concentration of 1 μM and samples were incubated for further 15 minutes (the choice of incubation times and substance concentrations was based on pilot experiments to provide the broadest possible representation of ANG I metabolite spectrum). Then, incubation buffer was removed and analyzed by LC/MS. Tissue fragments were dried overnight at 60°C and weighed to allow estimation of peptides' production per mg of dry tissue.

2.3.Chemicals

ANG II and ANG (1-7) were purchased from Sigma Chemicals (USA). Angiotensins: ANG I, ANG III, ANG IV and ANG (1-9) as well as ANG (1-5) were purchased from Bachem (USA). Perindoprilat was a gift from Servier (France). 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.

2.4.LC/MS measurement of angiotensin peptides

Metabolites of ANG I were analyzed by LC/MS method, developed in our previous studies (Bujak-Giżycka *et al.* 2007, Olszanecki *et al.* 2008), with analytical conditions optimized to current type of samples. Separation of peptides was performed on a reversed-phase HPLC system, equipped with a quaternary high pressure pump L-7000 (Merck, Germany), using a Purospher STAR RP C18e column (125 mm × 2 mm ID, 5 μm particle size) with an appropriate guard C18 column (4 mm × 4 mm ID, 5 μm particle size). Samples were injected onto chromatographic column in a volume of 50 μl. The optimized mobile phase solvents were: 5% acetonitrile in a buffer of 4 mM ammonium formate with 4 mM of formic acid (phase A) and 90% acetonitrile in the same buffer (phase B). Angiotensin peptides were separated at a flow rate of 0.25 ml/min with a linear gradient of B in A. Retention times were as follow: 13.00min (ANG (1-7)), 13.70min (ANG (1-5)), 14.00min (ANG (1-9)), 14.45min (ANG III), 14.80min (ANG II), 15.10min (ANG IV) and 15.35min (ANG I).

Mass spectrometric detection was performed using an LCQ ion-trap mass spectrometer (Finnigan, San Jose, USA), with an ESI source (electrospray). All experiments were carried out in the positive ion mode (ion spray voltage 5kV; capillary voltage 46V; capillary temperature 200°C, nitrogen flow rate 65 psi) and selective ion monitoring (SIM) mode, as previously described (Bujak-Giżycka *et al.* 2007). M/z values of monitored ions corresponded mainly to doubly- protonated molecules of all angiotensins, except the single - charged ions of ANG IV and ANG (1-5), and were the following: 450 for ANG (1-7) (MW=899,02), 466 for ANG III (MW=931,11), 524 for ANG II (MW=1046,19), 592 for ANG (1-9) (MW=1183,34), 649 for ANG I (MW=1296,51), 665 for ANG (1-5) (MW=664,76) and 775 for ANG IV (MW=774,92). Acquired data were analyzed by Xcalibur Software v. 1.2 (Fig.1).

Samples for calibration curves of each examined peptide (mixture of standards) were analyzed in the same mode as above. Concentrations of angiotensins were calculated using the standard calibration curves, constructed by linear regression analysis by plotting of peak area *vs.* angiotensin concentration. Calibration curves were prepared for each examined peptide at a concentration range of 20 pM - 100 nM.

2.5.Statistics

Concentrations of angiotensins were expressed as in pmol/mg dry tissue. All values in the figures and text are expressed as mean ± SD. Concentrations of studied analytes were compared using non-parametric Wilcoxon's test. A *P* value of less than 0.05 was considered statistically significant.

3. RESULTS

Background level of all of examined angiotensinogen metabolites was negligible and didn't influence the results of further conversion of exogenously added ANG I.

After incubation of the renal arteries with ANG I, concentrations of produced metabolites [ANG II to ANG (1-5)] did not differ significantly between the rat strains, but were higher in 3M than in 7M animals ($p<0.04$) for ANG II, ANG III, and ANG IV metabolites), and higher in 7M rats for ANG (1-9) (Fig. 2 A-B).

The pattern of produced metabolites did not differ significantly between 7M and 3M rats, as well as between SHR and WKY strains. The main product of ANG I metabolism was ANG II, in all groups, but surprisingly, the higher production was observed in a control (WKY) group.

Pretreatment with perindoprilat caused very strong inhibition of ANG II production in WKY in comparison to SHR rats. In 3M animals, effect of ACE-I action was significantly more pronounced than in 7M rats. Concentration of ANG II was 88.88% lower in 3M WKY rats (0.10 pmol/mg after perindoprilat pretreatment vs. 0.90 pmol/mg), but in 7M animals decrease was only 60.27% (0.17pmol/mg vs. 0.42pmol/mg). Renal arteries of SHR rats were less sensitive to ACE-I action - in 3M group 56.83% decrease was observed (0.29pmol/mg vs. 0.67pmol/mg), and surprisingly, only 8.73% inhibition in 7M rats (0.30pmol/mg vs. 0.33pmol/mg) (Fig.3).

However, the most striking results obtained by us, concerned the level of ANG (1-9), produced by renal arteries after incubation with ANG I.

In 3M rats (Fig.4A), renal artery incubated with ANG I produced a similar amount of ANG (1-9) in both WKY and SHR rats. However, perindoprilat pretreatment of renal arteries, before ANG I incubation, almost tripled production of ANG (1-9) in SHR rats, while no increase or even some decline of ANG (1-9) production was evident in WKY rats ($p < 0.05$ comparing strains of 3M rats pretreated with perindoprilat).

In 7M SHR rats (Fig. 4B), production of ANG (1-9) after incubation of renal arteries with ANG I is increased, compared to 3M SHR rats. In 7M SHR rats, the concentration of ANG (1-9) is similar to the level observed in 3M rats pretreated with perindoprilat. In 7M SHR rats, pretreatment with perindoprilat does not induce further increase in ANG (1-9) production.

In contrast to SHR rats, renal arteries of 7M WKY rats produce from ANG I the similar amount of ANG (1-9) as 3M WKY rats (Fig. 4B). However, perindoprilat pretreatment in this group of 7M WKY rats leads to increase of ANG (1-9) production to the same level as in SHR rats. The influence of perindoprilat on production of ANG

II, ANG (1-7) and ANG (1-9) in both strains of 3M and 7M rats is summarized in Table 1.

4. DISCUSSION

In our study we assessed the metabolism of ANG I in renal arteries of spontaneously hypertensive rats. Apart from the dominant in the literature, classical pathway of metabolism to ANG II, ANG III, and ANG IV, an interesting alternative in the form of ANG (1-9), ANG (1-7), and ANG (1-5) was discovered. ANG (1-7) is an active peptide of RAS. It counteracts vasoconstriction by releasing nitric oxide and prostacyclin (Fang *et al.* 2013). Moreover, it opposes ANG II mitogenic, arrhythmogenic and procoagulant activities (Trask *et al.* 2007). Enhancing natriuresis and diuresis, it inhibits water and sodium retention caused by ANG II. Recently, it has been shown that vasodilatative and diuretic activities of ANG (1-7) are mediated *via* Mas, a G-protein coupled receptor (Santos *et al.* 2003). Furthermore, some activities of ANG (1-7) are blocked by AT_1 and AT_2 receptors antagonists (Clark *et al.* 2001). On the other hand, ANG (1-7) independently to *Mas* receptor, increases bradykinin activity and antagonizes hypertrophic action of ANG II (Su 2014).

Chronic activation of RAS is a major cause of chronic kidney diseases. Main components of RAS are present within the kidney (angiotensinogen, AT_1R, renin and ACE). There are many evidences of the important role of intrarenal ACE in inducing hypertension, even in the absence of systemic ACE (Gonzalez-Villalobos *et al.* 2013).

Our results showed that there are specific age related differences in metabolic pathways. In renal arteries of 3-month-old animals, higher amounts of ANG II, ANG III, and ANG IV were formed than in 7-month-old rats while ANG (1-9) was produced in lower level in these rats. Our most important finding is that angiotensin (1-9) is elevated in renal arteries isolated from 7-month-old SHRs after incubation with ANG I and also after treatment with perindoprilat.

Still very little is known about the role of ANG (1-9), other than being a precursor of ANG (1-7). Recent studies suggest that this peptide has beneficial effects in hypertension and organ damage, caused by ANG II. There are strong evidences that ANG (1-9) could reduce the hypertension and ameliorate cardiac and aortic wall hypertrophy and fibrosis in hypertensive rats (Ocaranza *et al.* 2014b). Treatment with ANG (1-9) resulted in decrease of circulating ANG II level and ACE activity.

ANG (1-9) is the RAS peptide found in the plasma of healthy volunteers and in patients treated with ACE-I (McKinney *et al.* 2014). It was also demonstrated that the concentration of ANG (1-9) increases in plasma and heart tissue of rats after myocardial infarction (Ocaranza *et al.* 2006). It can be produced from ANG I by carboxypeptidase A (CPA) or a CPA-like enzyme (McKinney *et al.* 2014, Ocaranza *et al.* 2014a) or by the recently recognized ACE2 (Vickers *et al.* 2002, Donoghue *et al.* 2000, Tipnis *et al.* 2000). Campbell *et al.* found that ANG (1-9) concentration in the kidney homogenates was higher than concentration of ANG II. Concentration of ANG (1-9) reached there about 50% of ANG I level (Campbell *et al.* 1991). Moreover, the main products of ANG I metabolism in human heart are both ANG (1-9) and ANG II, generated by heart chymase, ACE or carboxypeptidase A (Kokkonen *et al.* 1997).ANG (1-9) was shown to be a strong competitive inhibitor of ACE (at multiple-fold lesser concentration than

ANG I) both in human heart and platelets (McKinney *et al.* 2014, Ocaranza *et al.* 2014a). Moreover, ANG (1-9), like ANG (1-7) and ACEIs, increases nitric oxide and arachidonic acid release due to enhanced bradykinin action on its B_2 receptor (Jackman *et al.* 2002), while the links among vasodilator systems: NO, prostaglandins and bradykinin are already well known (Ocaranza *et al.* 2014a).

During the development of hypertension, changes in RAS components levels are observed. Results obtained from 5-week-old SHR rats (Zhou *et al.* 2012) showed that ANG II serum level increased rapidly for the first few weeks (starting from 5 to 11), then, up to 18 week, level of ANG II seems to achieve the steady state after the slight decrease. After 18^{th} week of age, ANG II level raised again. Interestingly, ANG (1-7) level showed the tendency to "*plateau-like*" until 14-week, then rapid increase was observed up to 17 week and next slight drop. Treatment with ACE-I resulted in significant decrease of ANG II production, but with low increase of ANG (1-7). Nevertheless, these results concern circulating RAS and cannot be directly translated to production in the tissue.

There are only few studies of ANG I metabolism in renal artery. According to our previous results, metabolism of ANG I in renal artery differs from aorta (Bujak-Giżycka *et al.* 2007). In rat aorta, main metabolite of ANG I was also ANG II, but its production was significantly higher than other metabolites. This fact indicates the prominent role of ACE-dependent metabolic pathway, whereas in renal arteries other metabolites were in comparable levels as ANG II, possibly due to high activity of others RAS enzymes, like ACE2 or NEP.

In our experiments, pretreatment with perindoprilat resulted in decrease in ANG II production by renal arteries ($p = 0.05$). This effect was strongly pronounced in 3-

month-old animals. Moreover, elevated production of ANG (1-7) and ANG (1-9) ($p = 0.05$) was observed in 3-month-old SHR rats, whereas in control group was negligible [Tab. 1].

Obtained results suggest that ACE-I caused not only expected decrease of ANG II formation, but also higher production of ANG (1-9) and more intensive metabolism of ANG II to ANG (1-7). Additionally, lower sensitivity of renal artery to ACE inhibition was observed in SHR rats at 7 months of age. Our data are in line with the results of Tikellis et al., (2006) who demonstrated that activity of ACE but not that of ACE2 is lower in 80 day old SHR rats compared to WKY controls and with the results of Mooradian & Lieberman (1990) who observed lower activity of ACE in 6- compared to 2-month old Fisher rats.

Quantitation of angiotensin peptides requires very specific and sensitive methods. In many studies, the most popular were radioimmunoassay (RIA) or ELISA measurements (Liu *et al.* 2011, Wang *et al.* 2003), but these methods do not allow to distinguish and assess each one of ANG I metabolites. This aim can be achieved by using liquid chromatography separation techniques (Singh *et al.* 2005), especially with mass spectrometric detection, like LC/MS (Olszanecki *et al.* 2008, Suski *et al.* 2013, Cui *et al.* 2007) or MALDI/TOF (Velez *et al.* 2009). We developed LC/MS method to measure angiotensin metabolism in *ex vivo* model (Fig. 1). This method was used in our studies, not only in animal tissue (Bujak-Giżycka *et al.* 2007, Olszanecki *et al.* 2008), but also in cell lines (Suski *et al.* 2013) and human tissue fragments (Stettner *et al.* 2013).

However, our study has got some limitations. *Ex vivo* model, used in present experiment, does not show the endogenous levels of ANG I peptides, but rather describes the "ability" of current tissue to metabolize ANG I. Similar models were successfully used by other authors (Velez *et al.* 2009, Singh *et al.* 2005) as well as in our earlier studies (Bujak-Giżycka *et al.* 2007, Olszanecki *et al.* 2008, Stettner *et al.* 2013) and we suppose that results are adequate to living tissue. On the other hand, it could be noticed that in this model, isolated tissue fragments present a static system, without influence of many biochemical pathways and without dynamic changes, characteristic for the living body.

In conclusion, we found that with ageing, renal artery of SHR rat become less sensitive to treatment with ACE-Is. Moreover, our observation that ANG (1-9) is elevated in renal arteries of 3M rats after ANG I and perindoprilat incubation in SHRs corroborates with the previous observations, pointing towards the role of ACE2 – ANG (1-7) - receptor *Mas* axis as an important endogenous mechanism of homeostasis in young rats, despite strong pressure from the opposite ACE - ANG II - AT_1 receptor axis (Chappell 2007). Confirmation of this mechanism could be obtained by experiments in *"gene-targeted"* mice without functional *Mas* receptor.

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REFERENCES:

BRAUN MC, HERRING SM, GOKUL N, MONITA M, BELL R, HICKS MJ, WENDERFER SE, DORIS PA. Hypertensive renal disease: susceptibility and resistance in inbred hypertensive rat lines. *J Hypertens* 31: 2050-9, 2013.

BUJAK-GIZYCKA B, MADEJ J, WOŁKOW PP, OLSZANECKI R, DRABIK L, RUTOWSKI J, KORBUT R: Measurement of angiotensin metabolites in organ bath and cell culture experiments by liquid chromatography - electrospray ionization - mass spectrometry (LC-ESI-MS). *J Physiol Pharmacol* **58**: 529-540, 2007.

CAMPBELL DJ, LAWRENCE AC, TOWRIE A, KLADIS A, VALENTIJN AJ**:** Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension* **18**: 763-**7**73**,** 1991.

CHAPPELL MC: Emerging evidence for a functional angiotensin-converting enzyme 2-angiotensin-(1-7)-MAS receptor axis: more than regulation of blood pressure? *Hypertension* **50**: 596-599, 2007.

CLARK MA, DIZ DI, TALLANT EA. Angiotensin-(1-7) downregulates the angiotensin II type 1 receptor in vascular smooth muscle cells. *Hypertension* **37**: 1141-1146, 2001.

CUI L, NITHIPATIKOM K, CAMPBELL WB: Simultaneous analysis of angiotensin peptides by LC-MS and LC-MS/MS: Metabolism by bovine adrenal endothelial cells. *Anal Biochem* **369**: 27-33, 2007.

DONOGHUE M, HSIEH F, BARONAS E, GODBOUT K, GOSSELIN M, STAGLIANO N, DONOVAN M, WOOLF B, ROBISON K, JEYASEELAN R, BREITBART RE, ACTON S: A novel angiotensin converting enzyme-related

carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* **87**: E1-E9, 2000.

FANG C, STAVROU E, SCHMAIER AA, GROBE N, MORRIS M, CHEN A, NIEMAN MT, ADAMS GN, LARUSCH G, ZHOU Y, BILODEAU ML, MAHDI F, WARNOCK M, SCHMAIER AH: Angiotensin 1-7 and Mas decrease thrombosis in Bdkrb2-/- mice by increasing NO and prostacyclin to reduce platelet spreading and glycoprotein VI activation. *Blood* **121**: 3023-3032, 2013.

FERRARIO CM: Addressing the theoretical and clinical advantages of combination therapy with inhibitors of the renin- angiotensin- aldosterone system: Antihypertensive effects and benefits beyond BP control. *Life Sci* **86**: 289-299, 2010.

FERRARIO CM, JESSUP J, CHAPPELL MC, AVERILL DB, BROSNIHAN KB, TALLANT EA, DIZ DI, GALLAGHER PE: Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation* **111**: 2605-2610, 2005.

FERREIRA AJ, MURÇA TM, FRAGA-SILVA RA, CASTRO CH, RAIZADA MK, SANTOS RA: New cardiovascular and pulmonary therapeutic strategies based on the Angiotensin-converting enzyme 2/angiotensin-(1-7)/mas receptor axis. *Int J Hypertens* **2012**: 147825, 2012.

GONZALEZ-VILLALOBOS RA, JANJOULIA T, FLETCHER NK, GIANI JF, NGUYEN MTX, RIQIUER-BRISON AD, SETH DM, FUCHS S, ELADARI D, PICARD N, BACHMANN S, DELPIRE E, PETI-PETERDI J, NAVAR LG, BERNSTEIN KE, MCDONOUGH AA: The absence of intrarenal ACE protects against hypertension. *J Clin Investig* **123**: 2011-2023, 2013.

IYER SN, FERRARIO CM, CHAPPELL MC: Angiotensin-(1-7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. *Hypertension* **31**: 356-361, 1998.

JACKMAN HL, MASSAD MG, SEKOSAN M, TAN F, BROVKOVYCH V, MARCIC BM, ERDÖS EG: Angiotensin 1-9 and 1-7 release in human heart: role of cathepsin A. *Hypertension* **39**: 976-981, 2002.

KOKKONEN JO, SAARINEN J, KOVANEN PT**:** Regulation of local angiotensin II formation in the human heart in the presence of interstitial fluid. Inhibition of chymase by protease inhibitors of interstitial fluid and of angiotensin-converting enzyme by Ang-(1-9) formed by heart carboxypeptidase A like activity. *Circulation* **95**: 1455-1463, 1997.

LIU CX, HU Q, WANG Y, ZHANG W, MA ZY, FENG JB, WANG R, DONG B, GAO F, ZHANG MX, ZHANG Y: Angiotensin- converting enzyme (ACE) 2 overexpression ameliorates glomerular injury in a rat model of diabetic nephropathy: a comparison with ACE inhibition. *Mol Med* **17**: 59-69, 2011.

LUQUE M, MARTIN P, MARTELL N, FERNANDEZ C, BROSNIHAN KB, FERRARIO CM: Effects of captopril related to increased levels of prostacyclin and angiotensin-(1-7) in essential hypertension. *J Hypertens* **14**: 799-805, 1996.

MA C, XIN H, JIANG XY, WANG YX, ZHANG YS: Relationship between renal injury and the antagonistic role of angiotensin- converting enzyme (ACE) and ACE2. *Genetics Mol Res* **13**: 2333-2342, 2014.

MCKINNEY CA, FATTAH C, LOUGHREY C, MILLIGAN G, NICKLIN SA: Angiotensin-(1-7) and angiotensin-(1-9): function in cardiac and vascular remodeling. *Clin Sci (Lond)* **126**: 815-827, 2014.

MOORADIAN AD, LIEBERMAN J: Age-related decrease in serum angiotensin converting enzyme activity: the role of thyroidal status and food intake. *J Gerontol* **45**: B24-7, 1990.

OBATA J, NAKAMURA T, TAKANO H, NAITO A, KIMURA H, YOSHIDA Y, SHIMIZU F, GUO DF, INAGAMI T: Increased gene expression of components of the renin- angiotensin system in glomeruli of genetically hypertensive rats. *J Hypertens* **18**: 1247-1255, 2000.

OCARANZA MP, GODOY I, JALIL JE, VARAS M, COLLANTES P, PINTO M, ROMAN M, RAMIREZ C, COPAJA M, DIAZ-ARAYA G, CASTRO P, LAVANDERO S: Enalapril attenuates downregulation of angiotensin-converting enzyme 2 in the late phase of ventricular dysfunction in myocardial infarcted rat. *Hypertension* **48**: 572-578**,** 2006.

OCARANZA MP, MICHEA L, CHIONG M, LAGOS CF, LAVANDERO S, JALIL JE: Recent insights and therapeutic perspectives of angiotensin-(1-9) in the cardiovascular system. *Clin Sci (Lond)* **127**: 549-557, 2014a.

OCARANZA MP, MOYA J, BARRIENTOS V, ALZAMORA R, HEVIA D, MORALES C, PINTO M, ESCUDERO N, GARCÍA L, NOVOA U, AYALA P, DÍAZ-ARAYA G, GODOY I, CHIONG M, LAVANDERO S, JALIL JE, MICHEA L: Angiotensin-(1-9) reverses experimental hypertension and cardiovascular damage by inhibition of the angiotensin converting enzyme/ Ang II axis. *J Hypertens* **32**: 771-783, 2014b.

OHNISHI K, MURASE M, NAKANO D, PELISH N, HITOMI H, KOBORI H, MORIMOTO S, MORI H, MASAKI T, OHMORI K, KOHNO M, ICHIHARA A,

NISHIYAMA A: Angiotensin- converting enzyme inhibitor does not suppress renal angiotensin II levels in angiotensin I infused rats. *J Pharmacol Sci*; **122**: 103-108, 2013.

OLSZANECKI R, BUJAK-GIŻYCKA B, MADEJ J, SUSKI M, WOŁKOW PP, JAWIEŃ J, KORBUT R: Kaempferol, but not resveratrol inhibits angiotensin converting enzyme. *J Physiol Pharmacol* **59**: 387-392, 2008.

RIBEIRO- OLIVEIRA A JR, NOGUIERA AI, PEREIRA RM, BOAS WW, DOS SANTOS RA, SIMOES E SILVA AC: The renin- angiotensin system and diabetes: an update. *Vasc Health Risk Manag* **4**:787-803, 2008.

SANTOS RA, FERREIRA AJ, VERANO-BRAGA T, BADER M: Angiotensin-converting enzyme 2, angiotensin-(1-7) and Mas: new players of the renin-angiotensin system. *J Endocrinol* **216**: R1-R17, 2013.

SANTOS RA, SIMOES E SILVA AC, MARIC C, SILVA DM, MACHADO RP, DE BUHR I, HERINGER-WALTHER S, PINHEIRO SV, LOPES MT, BADER M, MENDES EP, LEMOS VS, CAMPAGNOLE-SANTOS MJ, SCHULTHEISS HP, SPETH R, WALTHER T: Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* **100**: 8258-8263, 2003.

SINGH R, SINGH AK, LEEHEY DJ: A novel mechanism for angiotensin II formation in streptozotocin- diabetic rat glomeruli. *Am J Physiol Renal Physiol* **288**: F1183-F1190, 2005.

SOLER MJ, WYSOCKI J, BATTLE D: Angiotensin- converting enzyme 2 and the kidney. *Exp Physiol* **93**: 549-556, 2008.

STETTNER D, BUJAK- GIŻYCKA B, OLSZANECKI R, RYTLEWSKI K, HURAS H, KORBUT R: Assessment of angiotensin I metabolism in the human placenta using an LC/MS method. *Folia Med Cracov* **53**: 31-39, 2013.

SU JB: Different cross- talk sites between the renin- angiotensin and the kallikrein-kinin systems. *J Renin Angiotensin Aldosterone Syst* **15**: 319-328, 2014.

SUSKI M, GĘBSKA A, OLSZANECKI R, STACHOWICZ A, URACZ D, MADEJ J, KORBUT R: Influence of atorvastatin on angiotensin I metabolism in resting and TNF-α- activated rat vascular smooth muscle cells. *J Renin Angiotensin Aldosterone Syst* **15**: 378-383, 2014.

TIKELLIS C, COOPER ME, BIALKOWSKI K, JOHNSTON CI, BURNS WC, LEW RA, SMITH AI, THOMAS MC: Developmental expression of ACE2 in the SHR kidney: a role in hypertension? *Kidney Int.* **70**: 34-41, 2006.

TIPNIS SR, HOOPER NM, HYDE R, KARRAN E, CHRISTIE G, TURNER AJ: A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril insensitive carboxypeptidase. *J Biol Chem* **275**: 33238-33243, 2000.

TRASK AJ, FERRARIO CM: Angiotensin-(1-7): pharmacology and new perspectives in cardiovascular treatment. *Cardiovasc Drug Rev* **25**: 162-174, 2007.

UEHARA Y, MIURA S, YAHIRO E, SAKU K: Non-ACE pathway-induced angiotensin II production. *Curr Pharm Des* **19**: 3054-3059, 2013.

VELEZ JC, RYAN KJ, HARBESON CE, BLAND AM, BUDISAVLJEVIC MN, ARTHUR JM, FITZGIBBON WR, RAYMOND JR, JANECH MG: Angiotensin I is largely converted to Angiotensin (1-7) and Angiotensin (2-10) by isolated rat glomeruli. *Hypertension* **53**: 790-797, 2009.

VICKERS C, HALES P, KAUSHIK V, DICK L, GAVIN J, TANG J, GODBOUT K, PARSONS T, BARONAS E, HSIEH F, ACTON S, PATANE M, NICHOLS A, TUMMINO P: Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* **277**: 14838-14843, 2002.

WANG M, TAKAGI G, ASAI K, RESUELLO RG, NATIVIDAD FF, VATNER DE, VATNER SF, LAKATTA EG: Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. *Hypertension* **41**: 1308-1316, 2003.

ZHOU X, SHANG D, ZHANG T, LI L, ZHOU T, LU W: Modeling of angiotensin II-angiotensin-(1-7) counterbalance in disease progression in spontaneously hypertensive rats treated with/ without perindopril. *Pharmacol Res* **66**: 177-184, 2012.

ZHUO JL, FERRAO FM, ZHENG Y, LI XC: New frontiers in the intrarenal renin-angiotensin system: a critical review of classical and new paradigms. *Front Endocrinol (Lausanne)* **4**: 166, 2013.

Tables.

Table 1.

Concentrations of Angiotensin II (ANG II), Angiotensin (1-7) (ANG (1-7)) and Angiotensin (1-9) (ANG (1-9)) [pmol/mg of dry tissue] after incubation of renal arteries of 3-months (3M) and 7-months (7M) old Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats with 1 μM Angiotensin I (Mean ± Standard Deviation, pmol/mg of dry tissue). P – Renal arteries were pretreated with 10 μM perindoprilat before addition of ANG I. N – no pretreatment with perindoprilat.

Age group	Rat strain	ANG II		ANG (1-7)		ANG (1-9)	
		N	P	N	P	N	P
3M	SHR	0.67 ±0.33	0.29 ±0.11	0.37 ±0.20	0.63 ±0.17	0.13 ±0.09	0.34 ±0.19
	WKY	0.90 ±0.63	0.10 ±0.01	0.45 ±0.38	0.43 ±0.26	0.14 ±0.14	0.06 ±0.02
7M	SHR	0.33 ±0.34	0.30 ±0.38	0.32 ±0.27	0.27 ±0.26	0.30 ±0.17	0.32 ±0.22
	WKY	0.42 ±0.43	0.17 ±0.10	0.26 ±0.21	0.38 ±0.39	0.18 ±0.11	0.32 ±0.26

Figures.

Fig. 1.

Representative chromatograms of products of ANG I conversion by renal artery of SHR (A) and WKY (B) rats. Inserts show extracted values of monitored ions of major metabolites.

Fig. 2.

Production of ANG I metabolites (pmol/mg of dry tissue, mean ± SD) in response to 15 min incubation of renal artery fragments from WKY and SHR rats with angiotensin I (1 μM). A – 3 month old rats, B – 7 month old rats.

Fig. 3.

Decrease of ANG II (pmol/mg of dry tissue, mean ± SD) production in renal arteries incubated with ANG I (1μM) and pretreated (P) or not (N) with perindoprilat (10μM) in 3 month and 7 month old WKY and SHR rats.

Fig. 4.

Effect of perindoprilat (10μM) pretreatment (P) or no pretreatment (N) on ANG (1-9) concentration in ANG I (1μM) incubated renal arteries of 3month old (A) and 7 month old (B) WKY and SHR rats (pmol/mg of dry tissue, mean ± SD). *$p<0.05$

Fig. 1

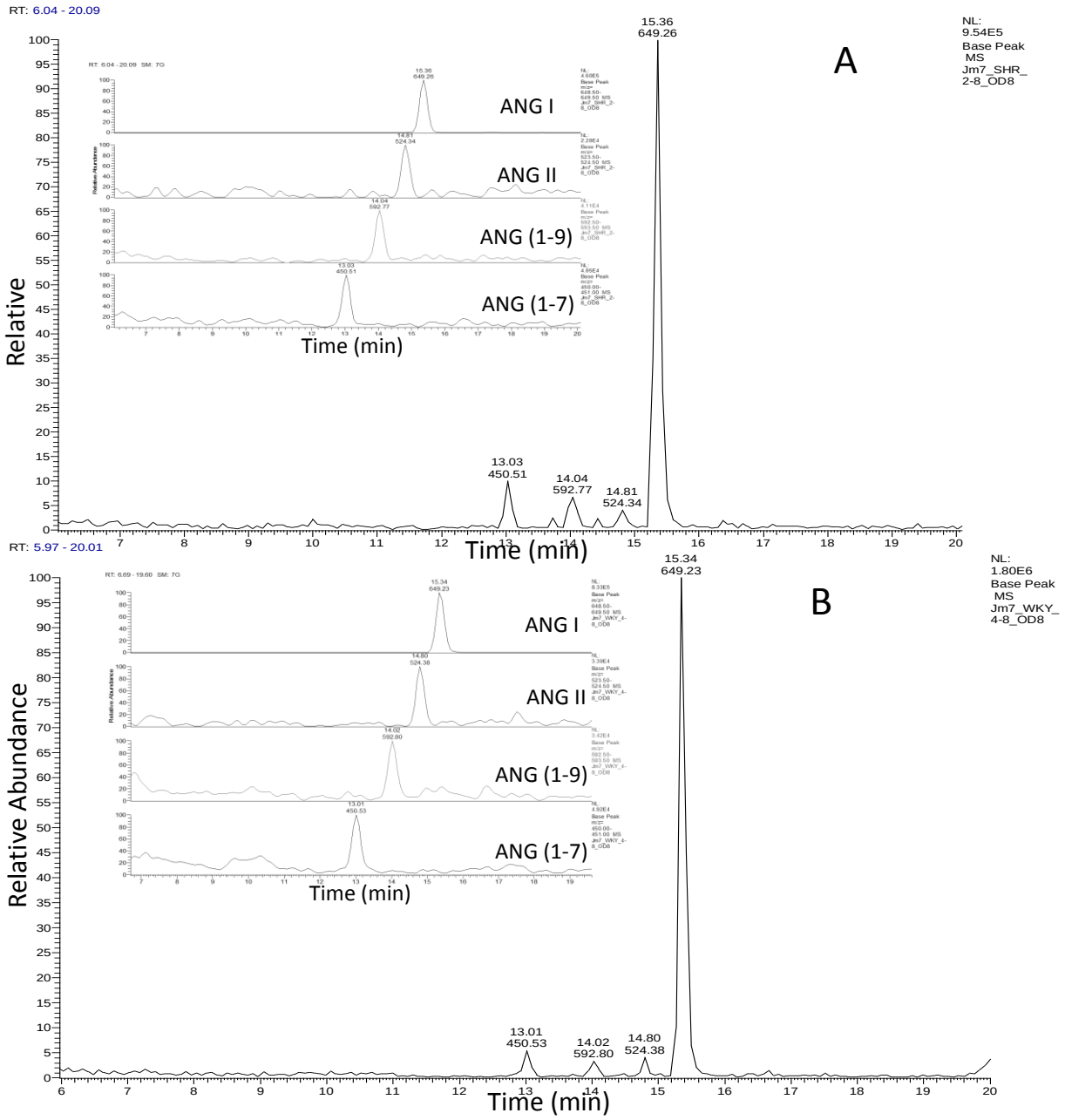

RT: 6.04 - 20.09
A
NL:
9.54E5
Base Peak
MS
Jm7_SHR_
2-8_OD8
15.36
649.26
ANG I
ANG II
ANG (1-9)
ANG (1-7)
Relative
Time (min)
13.03
450.51
14.04
592.77
14.81
524.34
RT: 5.97 - 20.01
B
NL:
1.80E6
Base Peak
MS
Jm7_WKY_
4-8_OD8
15.34
649.23
Relative Abundance
13.01
450.53
14.02
592.80
14.80
524.38

Fig. 2.

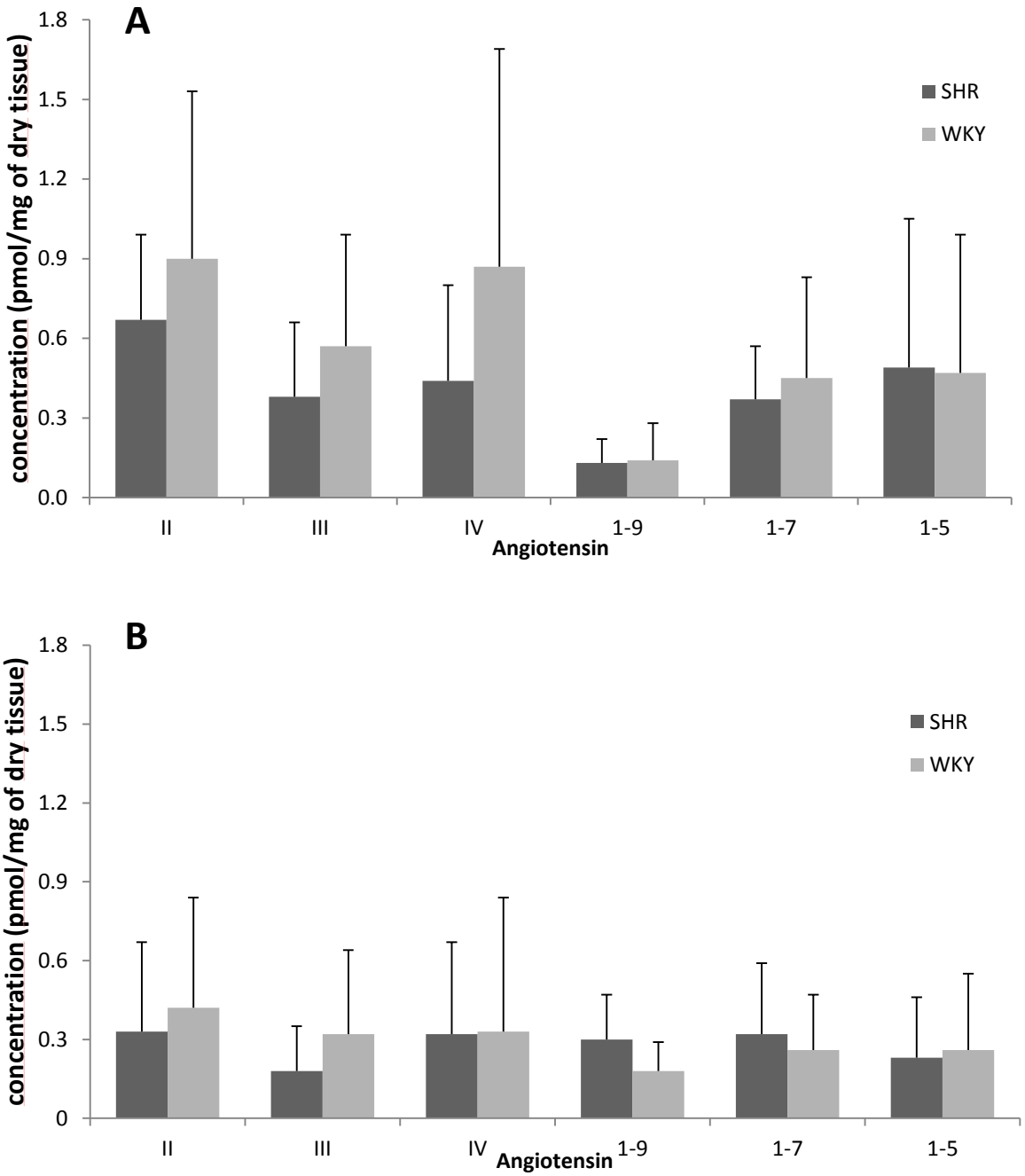

A
concentration (pmol/mg of dry tissue)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
SHR
WKY
II
III
IV
1-9
1-7
1-5
Angiotensin
B
concentration (pmol/mg of dry tissue)
1.8
1.5
1.2
0.9
0.6
0.3
0
SHR
WKY
II
III
IV
1-9
1-7
1-5
Angiotensin

Fig. 3.

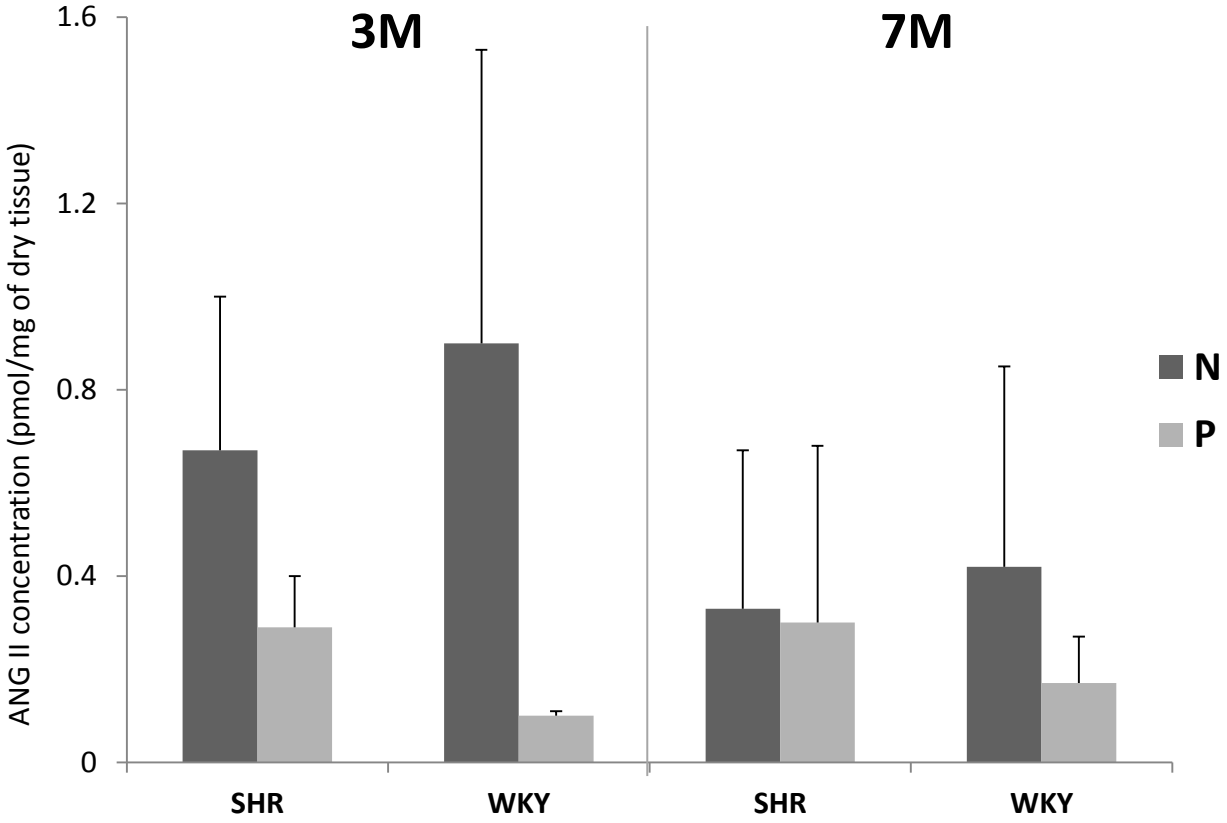
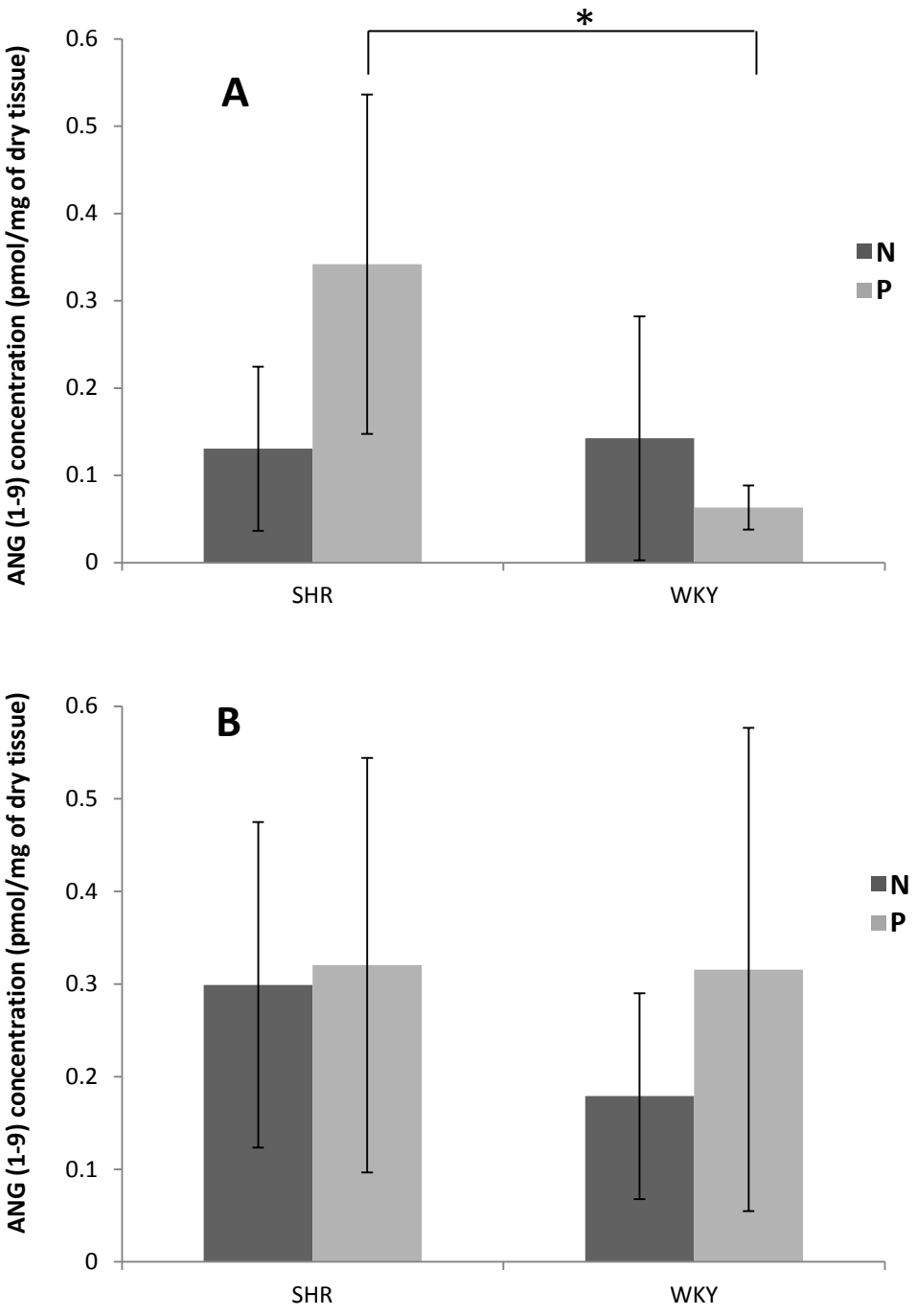

3M
7M
1.6
1.2
0.8
0.4
0
ANG II concentration (pmol/mg of dry tissue)
N
P
SHR
WKY
SHR
WKY

Fig. 4.


*
A
ANG (1-9) concentration (pmol/mg of dry tissue)
0.6
0.5
0.4
0.3
0.2
0.1
0
N
P
SHR
WKY
B
ANG (1-9) concentration (pmol/mg of dry tissue)
0.6
0.5
0.4
0.3
0.2
0.1
0
N
P
SHR
WKY