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Rapid Communication

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KAEMPFEROL, BUT NOT RESVERATROL INHIBITS ANGIOTENSIN CONVERTING ENZYME

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Inhibition of angiotensin converting enzyme (ACE) has proved to be beneficial in the treatment of various cardiovascular disorders. The aim of this study was to evaluate ACE inhibitory potential of two polyphenolic compounds with different structures: resveratrol (present in high quantities in French wine) and kaempferol (abundant in greens), using method of liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) for ex vivo measurement of angiotensin I to angiotensin II conversion by ACE in aortic tissue of Wistar-Kyoto rats. In this setting, kaempferol (10-30-100 μM), but not resveratrol (10-30-100 μM) appeared to inhibit dose-dependently conversion of Ang I to Ang II. Although the mechanism of ACE inhibition by kaempferol remains to be elucidated, this observation may help in search or designing of new classes of ACE inhibitors.

Key words: *angiotensin converting enzyme (ACE), ACE inhibitors (ACEi), resveratrol, flavonoids, French wine, hypertension*

INTRODUCTION

Inhibition of angiotensin converting enzyme (ACE) has proved to be effective strategy in the treatment of broad spectrum of cardiovascular disorders (1, 2). Since the development of the first orally active inhibitor of ACE - captopril (1975), the growing number of its congeners, namely ACE inhibitors (ACEi), became mainstay in the therapy of hypertension, heart failure, coronary heart disease and kidney disorders (3). However, as the classical ACEi are showing

well described adverse effects (3), there is constant interest in search of new, safe compounds with ACE inhibitory potential.

It has been demonstrated that polyphenols of plant origin possess biological actions, which could be of potential benefit in prevention and treatment of cardiovascular disorders (4 - 7). Indeed, epidemiological studies have suggested that the low incidence of coronary heart disease in the French and other Mediterranean populations, despite a diet rich in saturated fat, can be attributed to the high rate of polyphenol consumption derived from wine, tea or various plant foods (8). It is tempting to speculate that part of the beneficial effects of these compounds might depend on the influence on angiotensin metabolism.

The aim of this study was to evaluate ACE inhibitory potential of two polyphenolic compounds with different structures (*Fig. 2*): resveratrol - present in high quantities in French wine (9) and kaempferol, abundant in greens (4) - using method of liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) for measurement of angiotensin I to angiotensin II conversion by ACE in rat aortic tissue.

MATERIALS AND METHODS

Isolation and treatment of rat tissues

Male Wistar-Kyoto rats at either 3 months of age and 300-380 g of weight or 7 months of age and 350-420 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 (glucose 10 mM, pyruvate 2 mM, HEPES 10 mM, EDTA 0.03 mM, NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 15 mM) and cleaned of thrombi and tissue remnants. Blood vessels were cut into a suitable number of rings and opened flat. Tissue fragments were incubated for 30 minutes at 37°C in Eppendorf tubes in 550 ml of Krebs-Henseleit solution and continuously bubbled with $95\%O_2/5\%CO_2$.

Sample of 50 µl of buffer was removed to provide information on background production of angiotensin metabolites. Then kaempferol, resveratrol (in both cases final concentrations: 10-30-100 µM, dissolved in dimethylsulphoxide), dimethylsuphoxide (as a control; final concentration: 0,002%) or perindoprilat (final concentration: 10 µM) were added to the tubes. After 5 min of incubation, angiotensin I was added (final concentration 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 with mass spectrometry. 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.

Liquid chromatography - electrospray ionization - mass spectrometry (LC-ESI-MS)

LC-ESI-MS measurements of angiotensin metabolites were done as described previously (10). Briefly, separation of angiotensins was performed on a reversed-phase, high performance liquid chromatography (HPLC) system using a Purospher STAR RP C18e column (125mm x 2mm ID,

5mm particle size). The mobile phase solvents were: 5% acetonitrile in a buffer of 4mM ammonium formate with 4 mM formic acid (phase A) and 90% acetonitrile in a buffer of 4mM ammonium formate with 4 mM formic acid (phase B). The angiotensins were separated at a flow rate of 0,25ml/min with a linear gradient. The mobile phase gradient was started with 100% of phase A isocratic for 2 min, followed by a linear increase from 0 to 40% phase B within 10 min, then decreasing to 20% B in 5 min, next increasing to 90% B over 5 min and 90% B isocratic for 2 min.

Mass spectrometric detection was performed using a LCQ ion- trap mass spectrometer (Finnigan, San Jose, USA), equipped with an ESI source (electrospray). All experiments were carried out in the positive ion mode. The main working parameters were as follows: nitrogen (sheath gas) flow rate 65psi, ion spray voltage 5kV, capillary temperature 200°C, capillary voltage 46V, tube lens offset 40V. For detection, selected ion monitoring (SIM) mode was used with a total microscans = 1 and maximum inject time of 500 ms. Scanned mass range was set at 450 - 760 Da. LCQ data were analyzed by using the Xcalibur Software (Finnigan, San Jose, USA). Concentrations of angiotensins (Ang I, II, III, IV, 1-9, 1-7, 1-5) were calculated using the standard calibration curves, constructed by linear regression analysis by plotting of peak area *vs*. angiotensin concentration and calculated as pmol/mg dry tissue.

Chemicals

Kaempferol (3,4',5,7-Tetrahydroxyflavone; 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), resveratrol (3,4',5-Trihydroxy-*trans*-stilbene 5-[(1E)-2-(4-Hydroxyphenyl)ethenyl] -1,3-benzenediol) and standards of Ang I, II, III, IV, 1-9, 1-7, 1-5 were purchased from Sigma (USA). Formic acid (99%) (Riedel de Haen, Germany), acetonitrile (Baker, USA), ammonium formate (Fluka, Germany) were HPLC grade. Deionized water was obtained using a MillQ system (MilliPore, USA).

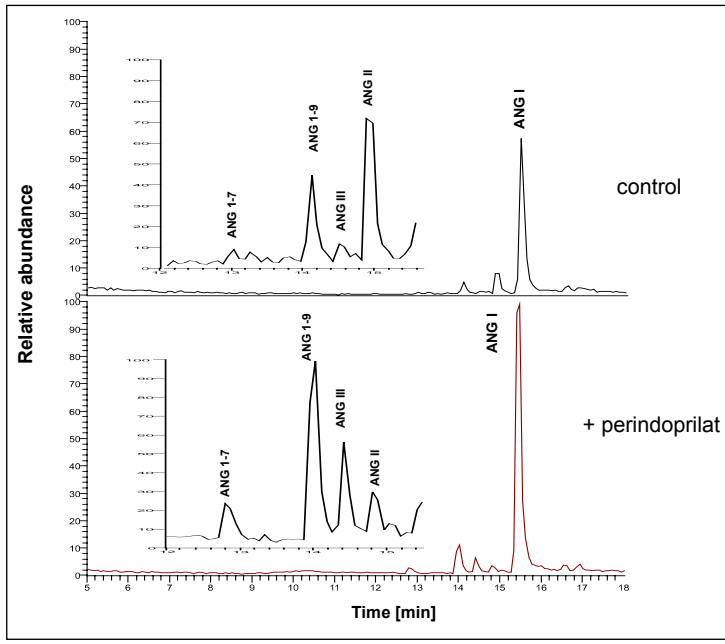

Fig. 1. Representative chromatograms of products of Ang I conversion by the rat aorta incubated for 15 minutes with Ang I (1µM) in the absence (upper panel) and the presence (lower panel) of perindoprilat (10µM). (Inserts: magnifications of chromatogram fragments of 12-15,5 min retention time).

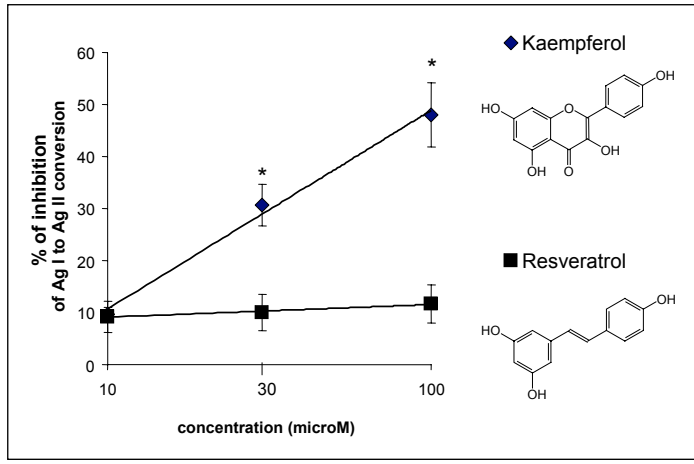

Fig. 2. Chemical structures of kaempferol and resveratrol and influence of both compounds on Ang I to Ang II conversion by rat aorta, expressed in semi-logarithmic scale as percent of inhibition vs. control (no inhibition) for n=4 experiments (± S.E.M).
* P<0,01 *vs*. control

Statistics

For each compound, data were expressed as % of inhibition of control Ag II formation and compared using non-parametric Wilcoxon's test. A P value less than 0,01 was considered to be statistically significant.

RESULTS

Incubation of Ang I with rat aorta resulted in production of high amounts of Ang II, slightly lower generation of Ang 1-9 and significantly low production of Ang III and Ang 1-7, (*Fig. 1*). ACE inhibitor, perindoprilat strongly inhibited formation of Ang II (80 ±16 *vs*. 834 ±79 pmol/mg tissue, p<0,01) and tend to increase of Ang 1-9 and Ang 1-7 generation by rat aortic tisue (*Fig. 1*).

Kaempferol, but not resveratrol dose-dependently inhibited formation of Ang II by rat aorta reaching level of 46% inhibition at the concentration of 100 μmol/l (*Fig. 2*).

DISCUSSION

Here, by LC-ESI-MS method, we identified kaempferol as inhibitor of conversion of Ang I to Ang II in rat aorta. The method of LC-ESI-MS has proved to be accurate and reproducible for comprehensive quantitation of angiotensin metabolites in organ bath of tissue fragments and in medium of cultured cells, exposed to Ang I for relatively short period of time (10, 11). Importantly, in organ bath of rat aortic tissue Ang I was converted to Ang II predominantly by ACE, as evidenced by strong inhibitory action of selective enzymatic inhibitor of ACE (perindoprilat). Thus, the model of LC-ESI-MS measurement of Ang I to Ang II

conversion by rat aorta seems to be viable for estimation of influence of exogenous compounds on ACE activity.

Interestingly, in this setting, another well-known phytochemical - resveratrol - did not show any activity. This difference might clearly depend on the different chemical structures of both compounds (*Fig. 2*). There were few reports pointing to ACE and/or neutral endopeptidase (NEP) inhibitory properties of some plant-derived polyphenols as well as showing preliminary structure-activity relationships in selected groups of compounds (procyanidins and flavons): the effect was generally dependent on the number of epicatechin units forming the procyanidin (12 - 16) and distribution of free hydroxyl groups in flavons (17). Our study may suggest that the ACE inhibitory activity of kaempferol may depend on the presence of pyran ring with carbonyl group (lacking in resveratrol), however, further studies are needed to clarify this question.

Our study was not designed to provide insight in mechanism of kaempferol inhibition of ACE. In fact, almost nothing is known about exact mechanism(s) of ACE inhibition by other polyphenols. Uchida et al. reported that the inhibition of ACE by congeners of flavonoids - condensed tannins - was reversible and non-competitive (18).

Another question is the potency of ACE inhibition by flavonoids. When compared to perindoprilat, kaempferol appeared to be rather weak ACE inhibitor. However, it should be noted that dietary intake of polyphenols could be as high as 1g/d (4). Whether dietary kaempferol could modulate ACE activity *in vivo*, this attractive hypothesis remains to be tested.

For many years the whole group of polyphenols were thought to protect the cells against oxidative damage as reactive oxygen species scavengers only, however, it is clear now that their biological effects extend well beyond antioxidant action (19). Extraordinary diversity and complexity of chemical structures implicate great variety of biological activities of these compounds. Our study revealed difference in ACE inhibitory action between two phytochemicals, common constituents of Mediterranean diet. This observation may help in search or designing of new and safe ACE inhibitors.

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REFERENCES

1. Atlas SA. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. *J Manag Care Pharm* 2007; 13(8 Suppl B): 9-20.
2. Kramkowski K, Mogielnicki A, Buczko W. The physiological significance of the alternative pathways of angiotensin II production. *J Physiol Pharmacol* 2006; 57: 529-539.

3. Wong J, Patel RA, Kowey PR. The clinical use of angiotensin-converting enzyme inhibitors. *Prog Cardiovasc Dis* 2004; 47: 116-130.
4. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 2002; 22: 19-34.
5. Ryszawa N, Kawczynska-Drozdz A, Pryjma J, *et al*. Effects of novel plant antioxidants on platelet superoxide production and aggregation in atherosclerosis. *J Physiol Pharmacol* 2006; 57: 611-626.
6. Dell'Agli M, Busciala A, Bosisio E. Vascular effects of wine polyphenols. *Cardiovasc Res* 2004; 63: 593-602.
7. Olszanecki R, Gebska A, Kozlovski VI, Gryglewski RJ. Flavonoids and nitric oxide synthase. *J Physiol Pharmacol* 2002; 53(4 Pt 1): 571-584.
8. Graf BA, Milbury PE, Blumberg JB. Flavonols, flavones, flavanones, and human health: epidemiological evidence. *J Med Food* 2005; 8: 281-290.
9. Pervaiz S. Resveratrol: from grapevines to mammalian biology. *FASEB J* 2003; 17:1975-1985.
10. Bujak-Gizycka B, Madej J, Wolkow PP, *et al*. Measurement of angiotensin metabolites in organ bath and cell culture experiments by liquid chromatography - electrospray ionization - mass spectrometry (LC-ESI-MS). *J Physiol Pharmacol* 2007; 58: 529-540.
11. 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* 2007; 369: 27-33.
12. Actis-Goretta L, Ottaviani JI, Keen CL, Fraga CG. Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. *FEBS Lett* 2003; 555: 597-600.
13. Ottaviani JI, Actis-Goretta L, Villordo JJ, Fraga CG. Procyanidin structure defines the extent and specificity of angiotensin I converting enzyme inhibition. *Biochimie* 2006; 88:359-365.
14. Loizzo MR, Said A, Tundis R, *et al*. Inhibition of angiotensin converting enzyme (ACE) by flavonoids isolated from Ailanthus excelsa (Roxb) (Simaroubaceae). *Phytother Res* 2007; 21: 32-36.
15. Oh H, Kang DG, Kwon JW, *et al*. Isolation of angiotensin converting enzyme (ACE) inhibitory flavonoids from Sedum sarmentosum. *Biol Pharm Bull* 2004; 27: 2035-2037.
16. Persson IA, Josefsson M, Persson K, Andersson RG. Tea flavanols inhibit angiotensin-converting enzyme activity and increase nitric oxide production in human endothelial cells. *J Pharm Pharmacol* 2006; 58: 1139-1144.
17. Bormann H, Melzig MF. Inhibition of metallopeptidases by flavonoids and related compounds. *Pharmazie* 2000; 55: 129-132.
18. Uchida S, Ikari N, Ohta H, *et al*. Inhibitory effects of condensed tannins on angiotensin converting enzyme. *Jpn J Pharmacol* 1987; 43: 242-246.
19. Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: antioxidants and beyond. *Am J Clin Nutr* 2005; 81(1 Suppl): 215S-217S.

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