Original Paper

Effects of AT1 Receptor Blockade on Plasma Thromboxane A₂ (TXA₂) Level and Skin Microcirculation in Young Healthy Women on Low Salt Diet

Ana Cavka^a Anita Cosic^a Ivana Grizelj^a Akos Koller^b Bojan Jelaković^c
Julian H Lombard^d Shane A Phillips^e Ines Drenjancevic^{a*}

^aDepartment of Physiology and Immunology, Faculty of Medicine, University of Josip Juraj Strossmayer in Osijek, Osijek, Croatia; ^bDepartment of Pathophysiology and Gerontology, Faculty of Medicine, and Szentagothai Research Center University of Pecs, Hungary; ^cSchool of Medicine University of Zagreb, Department for Nephrology, Hypertension, Dialysis and Transplantation, University Hospital Zagreb, Zagreb, Croatia; ^dDepartment of Physiology, Medical College of Wisconsin, Milwaukee, WI, USA; ^eDepartment of Physical Therapy, College of Applied Health Sciences, University of Illinois at Chicago, Chicago, IL, USA

Key Words

Microcirculation • Endothelium • Laser Doppler flowmetry • Renin-angiotensin system • Thromboxane A₂

Abstract

Objective: To determine the effect of AT1 receptor antagonism on skin microcirculation and plasma level of thromboxane A₂ (TXA₂). **Methods:** Healthy women (n=20) maintained 7 days low salt (LS) diet (intake <40 mmol Na/day) without (LS) or together with 50 mg/per day of losartan (a selective AT1 receptor inhibitor) (LS diet+losartan group). Laser Doppler flowmetry (LDF) measurements of changes in post occlusive hyperemic blood flow, plasma concentration of stable TXA₂ metabolite, thromboxane B₂ (TXB₂) and plasma renin activity (PRA), aldosterone concentration, electrolytes (Na⁺, K⁺), as well as blood pressure and heart rate were determined before and after study protocols. **Results:** PRA and aldosterone increased significantly after 7 days of both LS diet and LS diet+losartan. LS diet or LS diet+losartan administrations had no significant effect on post-occlusion hyperemia. While there was no change in TXB₂ after LS diet, TXB₂ significantly increased after one week of LS+losartan compared to control levels (cTXB₂ pg/mL control 101±80 vs. LS diet+losartan 190±116, p<0.05). **Conclusion:** These data suggest that inhibition of AT1 receptors could lead to activation of AT2 receptors, which maintain hyperemia, despite the increased level of vasoconstrictor TXA₂. These findings also suggest an important role of crosstalk between renin-angiotensin system (RAS) and arachidonic acid metabolites in the regulation of microcirculation under physiological conditions.

Copyright © 2013 S. Karger AG, Basel

Ines Drenjancevic, MD, PhD

Department of Physiology and Immunology, Faculty of Medicine, University of J.J. Strossmayer in Osijek, J. Huttlera 4, HR-31 000, Osijek (Croatia)
Tel. 0038531/512-800, Fax 0038531/512-866, E-Mail ines.drenjancevic@mefos.hr

Introduction

The RAS is a major homeostatic system that controls body fluid volume, electrolyte balance, blood pressure as well as neuronal and endocrine functions related to cardiovascular control and is critical for maintaining arteriolar structure and vascular reactivity [1]. The RAS exhibits its effects through its main effector molecule angiotensin II (ANG II), which binds to specific membrane-bound angiotensin receptors located in multiple tissues, including the vasculature [1-3].

At present, at least two ANG II receptors, AT1 and AT2, have been identified [4-6]. The majority of well-known ANG II actions, such as vasoconstriction, are mediated via AT1 receptor subtype stimulation. However, recent evidence suggests that the AT2 receptor not only opposes the effects of AT1 receptor, but also has unique effects beyond an interaction with AT1 receptor signaling, such as vasodilation [7]. AT1 receptor is expressed ubiquitously in blood vessels, whereas the AT2 receptor is expressed relatively weakly in large vessels, such as the aorta and at a much higher level in many resistance arteries and microvessels [8-10].

Our previous studies indicate the importance of normal level of circulating ANG II and the subsequent activation of AT1 receptors in the maintenance of normal dilator responses of microvessels and resistance arteries of normotensive rats [11-14] and in Dahl salt-sensitive rats that exhibit impaired vascular relaxation even when they are normotensive and fed with a low salt diet [15]. Treatment of normotensive rats with the AT1 receptor blocker losartan substantially alters the responses of isolated skeletal muscle resistance arteries in response to reduced PO_2 , possibly due to alterations in the release of the cyclooxygenase-dependent constrictor TXA_2 and the vasodilator prostaglandin PGI_2 from the endothelium. However, there was no change in ACh-induced dilation of the same blood vessels [16]. In other study, Phillips et al. demonstrated significantly altered ACh-induced vasodilation, while dilation to hypoxia was maintained in the middle cerebral arteries from rats chronically taking losartan [17]. Taken together, the results of these studies indicate that the normal functioning of the RAS and tonic activation of the AT1 receptor by ANG II are required to maintain normal responses to vasodilator stimuli in both skeletal and cerebral resistance arteries. Furthermore, these findings provide evidence that continuous AT1 receptor activation may be crucial for the normal synthesis of cyclooxygenase-dependent metabolites of arachidonic acid involved in normal vascular response to various stimuli.

Because some studies suggest that chronic RAS blockade improves endothelial function in hypertensive patients [18, 19] and that ANG II is crucial regulator of signaling mechanisms important for peripheral vascular function [12, 13, 20, 21], providing evidence for the role of the AT1 receptor in the maintenance of normal vascular function is of critical importance.

Until recently, all studies were conducted on males to "eliminate the confounding problem of cycling" and while the data obtained from these subjects have greatly advanced our knowledge of physiology and pathophysiology now we know, the consequences of such studies is limited view of the mechanisms controlling organ and tissue function and to limitations in the diagnosis and care of humans with disease [22].

It is known that sex disparities are present in incidence, prevalence, outcome, treatment and diagnosis of cardiovascular diseases. Some evidence even suggests that the classical risk factors for cardiovascular diseases may not apply to women, as they do in the men, particularly in regard to coronary calcification, diastolic heart failure and microvascular diseases [22, 23]. With these considerations in mind, we chose a population of healthy young women for our study in an effort to help elucidate the physiology and pathophysiology of the female vascular system.

The aim of the present study was to determine the effect of AT1 receptor antagonist losartan on skin microvascular reactive hyperemia and plasma level of cyclooxygenase vasoconstrictor products in young healthy women on a LS diet.

Materials and Methods

Study population

Healthy female medical students (age 20 ± 1) recruited by advertisement at the Faculty of Medicine University of Osijek participated in the study. Exclusion criteria included a history of hypertension, coronary artery disease, diabetes, hyperlipidemia, renal impairment, cerebrovascular and peripheral artery diseases. None of the subjects were taking drugs that could affect their cardiovascular system. Written informed consent was obtained from each subject. The study protocol and procedures conformed to the standards set by the latest revision of the *Declaration of Helsinki* and were approved by the Ethical Committee of the Faculty of Medicine, University of Osijek.

Study protocol

During the study all subjects were instructed to maintain a low-sodium diet, with intake of 40 mmol sodium per day (DASH diet, U.S. Department of Health and Human Services, 2006) for 7 days. Ten participants comprising LS diet+losartan group were given 50 mg of losartan (selective AT1 receptor inhibitor) per day during the 7 day diet protocol. Other ten participants on low-sodium diet presented LS diet group.

A venous blood sample was taken before and after the study protocol after 30 min resting in supine position. A 24 hour urine sample was collected before and after the study protocol. During the 24-hour collection, participants followed a standard procedure in which they began to collect urine after the first urination and continued through the first urination the next morning.

Blood pressure and heart rate (HR), measured at the beginning of each visit were determined after 15 minutes rest in seated position using a semiautomatic oscillometric monitor (OMRON). The final values of arterial blood pressure and heart rate were the mean of three repeated measurements.

The height and weight of each subject was measured at each visit to determine Body Mass Index (BMI), and the circumference of the hips and waist were measured to determine Waist to Hip Ratio (WHR).

Assessment of skin microcirculatory blood flow

Microcirculatory blood flow was assessed by noninvasive method LDF (MoorVMS-LDF, Axminster, UK). We have measured changes in blood flow during reactive hyperemia following release of an occlusion of blood flow. Numerous studies suggest that changes in cell shape, pressure, stretch, and flow/shear stress, as a result of occlusions activate mechanosensitive-signaling mechanisms in the arteriolar wall, which elicit reactive dilation that resembles the characteristics of reactive hyperemia. Thus, in addition to previously described metabolic factors, mechanosensitive mechanisms, via activation of endothelial and smooth muscle cell stretch receptors and endothelial NO synthesis, are likely to contribute significantly to the in vivo development of reactive hyperemia [24-27]. Reactive hyperemia is a complex microvascular response to an acute period of ischemia, in which the endothelium and its response to different endothelium derived relaxing and/or constricting factors (vascular reactivity) has a potential role [25]. Measurements were performed in a warm room (average temperature 23.5 ± 0.5 °C). Data collection started after 30 minutes acclimatization to avoid temperature-related changes in blood flow. During the measurement, the subjects were resting in the supine position. Because LDF measurements are very sensitive both to movement of the examinee and to movement of the probe or the wire attaching the probe to the LDF device, stationary position of the subjects was achieved by supporting frames that comfortably stabilized the subject's limb.

The laser probe was attached to subject's volar forearm skin 13-15 cm from the wrist with doubled sided adhesive discs provided by manufacturer. After 5 minutes of baseline measurement, vascular occlusion was induced by inflating a pneumatic cuff on the upper arm to 30 – 50 mmHg above the systolic blood pressure (SBP). Measurements were taken before, during, and after release of a 1 minute occlusion.

Changes in microcirculatory blood flow were expressed in arbitrary perfusion units (PU). To quantify relative changes of blood flow during post-occlusive hyperemia, the data were expressed as area under the curve (AUC) during baseline flow, occlusion and reperfusion. Results were expressed as the difference between the percentage of flow change during reperfusion and occlusion ($\Delta R-O$) in relation to baseline (Figure 1). All vascular measurements were performed by a single trained operator, blinded for the treatment group.

Fig. 1. Laser Doppler Flowmetry (LDF) measurement of skin microvascular blood flow. Changes in microcirculatory blood flow were expressed in arbitrary perfusion units (PU). Microcirculatory blood flow in a given time was determined by program calculating area under the curve (AUC) during baseline flow, occlusion and reperfusion (AUC is denoted by shaded portions of the trace). Since the flow does not reach the value of zero even when perfusion is absent, flow values are expressed as a percentage of a standard comparator. We calculated a percentage of flow during occlusion and reperfusion in relation to baseline flow. Final result was expressed as the difference between the percentage of flow change during reperfusion and occlusion (delta R-O) in relation to baseline. (Source: original trace, Laboratory for Vascular Physiology Department of Physiology and Immunology Faculty of Medicine Osijek).

Laboratory testing

Blood samples were analyzed for plasma electrolytes (sodium and potassium), plasma urea and plasma creatinine levels. Twenty-four hour urine samples were analyzed for sodium, potassium, urea, creatinine and albumin levels were performed at the Department of Clinical Laboratory Diagnostics, Clinical Hospital Centre Osijek. PRA and plasma aldosterone concentration were measured at the Clinical Institute of Nuclear Medicine and Radiation Protection, Clinical Hospital Centre Osijek, using standard radioimmunoassay techniques.

Measurement of plasma cyclooxygenase dependent vasoconstrictor thromboxane (TXA₂) levels

Because TXA₂ has a half life of only 37 seconds under physiological conditions, the production of TXA₂ in vivo is typically monitored by measurement of TXB₂ and 2,3-dinor TXB₂. TXB₂ is produced by the non-enzymatic hydration of TXA₂, and has been shown to be stable [28]. Plasma concentration of stable TXA₂ metabolite, TXB₂ was evaluated with the use of commercially available enzyme immunoassay kits purchased from Abnova (Taiwan) in the Laboratory for Vascular Physiology at the Department of Physiology and Immunology, Faculty of Medicine University of Osijek.

Statistical Analysis

All results are presented as mean ± SD. Clinical characteristics before and after the study protocol was compared by paired t-test. The Wilcoxon rank-sum test was used when variables were not normally distributed. A Student t-test was used to compare parameters between experimental protocols. When variables were not normally distributed, the Mann-Whitney Rank Sum Test was used. Statistical significance was set at P<0.05. In order to achieve 80% power and p<0.05 level of significance, paired t-test sample size analysis was used. That analysis established that a sample size of 20 participants was required and appropriate. SigmaPlot (version 11.2, Systat Software, Inc, Chicago, USA) program was used for statistical analysis.

Table 1. Hemodynamic Data of Study Population

Variable	before LS diet	after LS diet	before LS diet+losartan	after LS diet+losartan
SBP	106±9	101±9*	103±7	100±6
DBP	68±6	65±6*	67±6	66±6
MABP	80±6	77±5*	79±6	77±5
HR	74±10	76±14	82±12	81±15

* P<0.05; Results are expressed as mean±SD; LS - Low Salt, SBP - Systolic Blood Pressure, DBP - Diastolic Blood Pressure; MABP - Mean Arterial Blood Pressure, HR - Heart Rate

Table 2. Biochemical Parameters

Variable	before LS diet	after LS diet	before LS diet+losartan	after LS diet+losartan
24h urine volume, mL	1225±672	1105±516	1338±414	1280±361
24h urine sodium, mmol/dU	120±77	80±31*	160±65	75±43*
24h urine potassium, mmol/dU	42±13	37±9	40±11	34±10
24h urine urea, mmol/dU	237±76	186±62	236±86	175±75*
24h urine creatinine, µmol/dU	9913±3730	8926±3122	10871±2035	9879±3328
24h urine albumine, /dU	8±5	5±4*	6±2	3±1*
plasma sodium, mmol/L	137±1	136±1	137±2	136±2
serum potassium, mmol/L	4.2±0.4	4.2±0.4	4.1±0.5	4±0.4
serum urea, mmol/L	4.3±1	4.1±1	4.1±1	3.6±0.6*
serum creatinine, µmol/L	65±8	69±9*	64±6	66±8
PRA, ng/mL/h	0.6±0.2	1.2±0.7*	0.7±0.5	4.1±1.5*
plasma aldosterone, pmol/L	598±338	1150±1011*	434±186	733±297*

Results are expressed as mean±SD; LS - low Salt, PRA - Plasma Renin Activity; * P<0.05 before vs. after LS diet (n=10) or before vs. after LSD+losartan (n=10)

Results

Subject profiles

Twenty female subjects (mean age 20±1 years) participated in the study. Their BMI was 22.9±4.1 kg/m² and WHR was 0.70±0.03. The body weight of the subjects did not change after the study protocol compared to the control values. Table 1 summarizes the hemodynamic data of the study population. The mean value of three blood pressure measurements confirmed that the participants were normotensive before the study protocol. SBP, diastolic blood pressure (DBP) and mean arterial blood pressure (MABP) significantly decreased in LS diet group after diet protocol when compared to control values. There was no difference in SBP, DBP, MABP and HR between study groups both before and after study protocols.

Biochemical Parameters

Table 2 summarizes the changes in biochemical parameters measured in venous blood sample and 24 hour urine samples from young healthy women subjected to one week of LS diet or one week of LS diet and losartan administration. Plasma urea levels decreased after the LS diet+losartan protocol compared to control levels before study and serum creatinine levels increased after LS diet protocol when compared to control values before study protocol. Both PRA and plasma aldosterone levels increased significantly after 7 days of LS diet and LS diet+losartan intake when compared to control values before study protocols. Urinary sodium excretion and 24h urine sample albumin concentrations fell significantly after both study protocols when compared to control values before study protocols. Urinary urea excretion significantly fell after LS diet+losartan protocol. There was no statistically significant difference when comparing measured biochemical parameters between LS diet and LS diet+losartan group after 7 days of both study protocols.

Fig. 2. Effect of 7 days of low sodium diet protocol on skin reactive hyperemia without (2a) or with losartan administration (2b). Data are expressed as mean $\pm$ SD; R-O=change of microvascular blood flow between reperfusion and occlusion (in relation to baseline).

Fig. 3. Effect of 7 days of low sodium diet protocol on TXB₂ cyclooxygenase-dependent vasoconstrictor plasma levels without (3a) or with losartan administration (3b). * P=0.043 cTXB₂ before LS diet+losartan vs. after LS diet+losartan. Data are expressed as mean $\pm$ SD.

Effect of losartan on skin microcirculatory blood flow and plasma TXA₂ Levels

Figure 2 summarizes the effect of 7 days LS diet protocol with or without AT1 receptor antagonist losartan treatment on skin reactive hyperemia in young healthy women. Hyperemic blood flow following 1 minute of vascular occlusion, as assessed by LDF, was not significantly different after LS diet protocol vs. control values before the LS diet (delta R-O control before LS diet 1.2 $\pm$ 0.3 vs. after LS diet 1.3 $\pm$ 0.3, P=0.562) (Figure 2a), just as after one week of losartan administration during the LS diet protocol when compared to control (delta R-O control 1.3 $\pm$ 0.2 vs. LS diet+losartan 1.4 $\pm$ 0.2, P=0.734) (Figure 2b). Figure 3 summarizes the effect of LS diet protocol (Figure 3a) and losartan administration during LS diet protocol (Figure 3b) on plasma TXB₂ (a metabolite of TXA₂) concentrations. There was no change in TXB₂ plasma levels after LS diet protocol compared to control values (cTXB₂ pg/mL control before LS diet 108 $\pm$ 58 vs. after LS diet 95 $\pm$ 92, P=0.713), while plasma levels of TXB₂ increased significantly after one week of LS diet+losartan compared to control levels before LS diet+losartan (cTXB₂ pg/mL control before LS diet+losartan 101 $\pm$ 80 vs. after LS diet+losartan 190 $\pm$ 116, P=0.043). There was no statistically significant difference when comparing plasma TXB₂ levels between LS diet and LS diet+losartan group after 7 days of both study protocols (cTXB₂ pg/mL after LS diet 95 $\pm$ 92 vs. after LS diet+losartan 190 $\pm$ 116, P=0.194).

Discussion

The salient finding of the present study is that one week of AT1 receptor blockade in young healthy women on a low sodium diet significantly increased plasma levels of TXB₂, a stable metabolite of TXA₂ (Figure 3b) without causing significant changes in arterial blood pressure (Table 1) and with no effect on post-occlusion hyperemic blood flow of skin microcirculation (Figure 2b). However, such increase in plasma levels of TXB₂ could not be detected in young healthy women on low sodium diet (3a) suggesting that AT1 receptor blockade may play important role in regulation of cyclooxygenase-dependent pathway of metabolism of arachidonic acid.

It should be noted that this is the first study on the human model which confirmed the results of the study by Phillips et al. showing that losartan administration in rats increased plasma levels of cyclooxygenase-dependent vasoconstrictor TXA₂ without changes in skeletal muscle vascular responses to ACh (endothelium dependent dilation) and/or sodium nitroprusside (endothelium independent dilation) [16].

Recent studies have emphasized the importance of the RAS in maintaining normal vascular responses to various physiological stimuli, an effect that is mediated via activation of AT1 receptors [10-16]. However, because the most of the studies on the influence of AT1 blockade on vascular function are performed on a population of patients with high blood pressure and increased risk of cardiovascular complications, the pathways and mechanisms by which ANG II and/or AT1 receptor activation and/or inhibition may alter vascular reactivity and vascular function in physiological conditions, are not well understood. Moreover, recent studies suggest that AT1 receptor blockade may have disparate effects in healthy versus metabolic syndrome or hypertensive conditions [29].

Because a LS diet per se stimulates renin secretion and Ang II formation, we wondered what would occur if the formation of Ang II was blocked concomitant to a LS diet.

In the present study SBP, DBP and MABP significantly decreased after LS diet protocol and tended to decrease after LS diet+losartan protocol without changes between groups. This is consistent with the results of studies that examining the impact of the DASH diet on blood pressure in normotensive individuals [30]. Furthermore, both PRA and plasma aldosterone levels increased significantly after one week of both LS diet and LS diet+losartan protocols (Table 2). While elevated PRA is expected as a normal physiological response to low sodium intake during the study protocol, the anticipated effect of AT1 receptor blockade is to decrease plasma aldosterone levels. It is thought that ANG II stimulates aldosterone synthesis and secretion via AT1 receptor stimulation in the adrenal cortex [31]. In contrast, recent studies have demonstrated that selective antagonism of the AT1 receptor is associated with a substantial increase in plasma ANG II levels which may stimulate aldosterone secretion through the AT2 receptor when AT1 receptor is blocked – a phenomenon called aldosterone breakthrough during AT1 receptor blockade [31]. This is a likely explanation for the elevated plasma aldosterone levels that was found in present study. Also, in the present study one week of LS diet or LS diet+losartan decreased urinary albumin concentrations, which might be interpreted as protection of the endothelium from damage. This is an interesting finding, because recent studies have demonstrated that modest salt reduction reduced urine protein excretion in hypertensive population [32, 33]. In addition, some studies suggested that drugs that block the RAS may have preferential effects to lower proteinuria [33]. Recent studies on animal models have suggested that ANG II, maintains the normal reactivity to vasodilator stimuli in arterioles and resistance arteries [12, 34-39]. In rats, the impaired responses to vasodilator agonists after short-term exposure to a high-salt diet can be prevented by maintaining circulating levels of ANG II, suggesting that ANG II protected the mechanisms responsible for vascular relaxation in these vessels [12] which is mediated by interaction of ANG II with the AT1 receptor subtype [13]. Furthermore, losartan treatment of normotensive rats on normal-salt diet with losartan significantly altered the response of skeletal muscle resistance arteries to acute reduction in PO₂ [16]. This paradoxical vasoconstriction of resistance arteries to hypoxia after AT1 receptor blockade by losartan seems to be due to alterations in the release of the cyclooxygenase dependent vasoconstrictor TXA₂ from the endothelium [16].

Fig. 4. Possible role of AT₁ receptor blockade in regulating plasma TXA₂ levels and microcirculatory blood flow in young healthy female subjects on LS diet. Potential activation of AT₂ receptors during AT₁ receptor blockade may have an important role in maintaining vasodilator mechanisms. Another possible pathway involves potential interaction between AT₁ receptor blockade and the metabolism of thromboxane A₂ by AT₁ receptor blockade preventing the TXA₂ to interact with its receptors, thus maintaining normal microcirculatory blood flow despite increased plasma concentration of TXB₂.

However, there are no studies investigating this issue in human models, so the role of ANG II and activation of its AT₁ and AT₂ receptors on vascular reactivity during dietary salt modulation in healthy human individuals remains to be clarified. To investigate this question, participants in present study were subjected to one week of low salt diet without or with losartan administration. Decreased urinary sodium levels confirmed that experimental protocol was conducted consistently, and that the experimental subjects conformed to the diet guidelines. PRA also changed in the expected directions, providing additional confirmation of proper adherence to the dietary guidelines specified in this study (Table 2). Interestingly, we found that one week of losartan administration during the LS diet protocol, just as only LS diet protocol, did not induce any change in hyperemic tissue blood flow following 1 minute vascular occlusion. Whereas, one week of AT₁ receptor blockade significantly increased plasma levels of the cyclooxygenase dependent vasoconstrictor TXA₂. These results are consistent with previous studies on animal models, in which the hypoxic vasoconstriction of arteries from losartan-treated rats was blocked by indomethacin, indicating that a cyclooxygenase-derived constrictor product was responsible for the altered response to reduced PO₂ in those animals [16]. The most likely source of TXA₂ is the endothelium because cyclooxygenase-1 and -2 expressions are up to 20 times greater in the endothelium than in the smooth muscle cells [39].

One of the plausible explanations, why we did not observe a significant change in post occlusive hyperemic blood flow after losartan administration during LS diet, is activation of ANG II AT₂ receptors. In addition to their effect on blood pressure, long-term administration of AT₁ receptor blockers results in a several-fold increase in plasma ANG II and thus a possible overstimulation of AT₂ receptors [40-43]. Therefore, the effect of AT₂ receptor stimulation is becoming increasingly important in both physiological and pathological situations,

for example, hypertension. Furthermore, a cross-talk between the two receptor subtypes (AT1 and AT2) has been described [42, 43], and AT1 receptor blockade might also unmask potential effects of AT2 receptors that were overridden by AT1 receptor stimulation. This potential activation of AT2 receptors during AT1 receptor blockade may have an important role in maintaining vascular relaxation mechanisms, as previous studies have demonstrated that AT2 receptors play a vasodilator role and that selective stimulation of AT2 receptors in the presence of AT1 receptor antagonists is predicted to have a beneficial clinical effect in controlling blood pressure [44-46]. Other studies have shown that AT2 receptors also have a role in the control of the vascular tone by flow (shear stress) and that activation of AT2 receptors is involved in flow-dependent dilation [46, 47].

The most important finding of the present study is that AT1 receptor blockade significantly increased plasma levels of TXB₂ in young healthy women on LS diet. These data are consistent with growing evidence that losartan has AT1 receptor-independent actions [48] primarily related to anti-inflammatory and anti-aggregatory mechanisms. Most studies regarding this issue were done in animal models. Some studies indicate that losartan may be involved in the metabolism of the cyclooxygenase-dependent constrictor TXA₂ by interacting with its receptor rather than upregulating its generation [49, 50]. For example, Valentin et al. have shown that losartan prevents thromboxane A₂/prostanoid (TP) receptor mediated increase in microvascular permeability in the rat [51]. The results of the present study are in agreement with the study of Valetin et al [51] suggesting that AT1 receptor blockade interacts with the metabolism of thromboxane A₂ by preventing the TXA₂ to interact with its receptors, thus microcirculatory blood flow remains normal despite increased plasma concentration of TXB₂ (a stable metabolite of TXA₂) in women on a LS diet+losartan protocol. Figure 4 summarizes the observed effects and possible pathways that might be responsible for increased plasma TXA₂ level but unchanged microvascular blood flow responsiveness due to one week AT1 receptor blockade in young, healthy female subjects on LS diet.

A limitation of the study was the relatively small sample size. Taking this into consideration we did paired t-test sample size analysis, which has shown that our sample size was appropriate to achieve 80% power and p<0.05 level of significance.

Conclusion

Our study demonstrated that one week of LS diet with oral losartan administration led to a significant increase in plasma levels of the cyclooxygenase dependent vasoconstrictor TXB₂ (a metabolite of TXA₂) without causing any changes in blood pressure and/or skin microvascular blood flow responsiveness. We propose that losartan exhibited its additional effect by blocking the thromboxane A₂/prostanoid (TP) receptors. In addition, one can not exclude the possible interaction of ANG II with AT2 receptors, which may underlie preserved microcirculatory blood flow responsiveness, a possibility waiting for exploration in human subjects, once AT2 antagonists are approved for human use.

Acknowledgements

This study was supported by a grant of the Ministry of Science, Education and Sports of the Republic of Croatia: Effects of oxygen on vascular function in health and disease #219-2160133-2034, SROP-4.2.2.A-11/1/KONV-2012-0024 and Hungarian Natl Sci Res Fund (OTKA) K 108444.

References

- 1 Drenjancevic-Peric I, Jelakovic B, Lombard JH, Kunert MP, Kibel A, Gros M: High-Salt Diet and Hypertension: Focus on the Renin-Angiotensin System. *Kidney Blood Press Res* 2011;4:1-11.
- 2 Baudouin M, Meyer P, Worcel M: Specific binding of 3H-angiotensin II in rabbit aorta. *Biochem Biophys Res Commun* 1971;42:434-440.

- 3 Devynck MA, Pernollet MG, Meyer P, Femandjian S, Fromageot P: Angiotensin receptors in smooth muscle cell membranes. *Nat New Biol* 1973;245:55-58.
- 4 Inagami T, Guo DF, Kitami Y: Molecular biology of angiotensin II receptors: an overview. *J Hypertens* 1994;12:S83-S94.
- 5 Matsubara H, Inada M: Molecular insights into angiotensin II type 1 and type 2 receptors: expression, signaling and physiological function and clinical application of its antagonists. *Endocr J* 1998;45:137-150.
- 6 Chrysant SG: The Role of Angiotensin II Receptors in Stroke Protection. *Curr Hypertens Rep* 2012;14:202-208.
- 7 Horiuchi M, Iwanami J, Mogi M: Regulation of angiotensin II receptors beyond the classical pathway. *Clin Sci (Lond)* 2012;123:193-203.
- 8 Viswanathan M, Tsutsumi K, Correa FMA, Saavedra JM: Changes in expression of angiotensin receptor subtypes in the rat aorta during development. *Biochem Biophys Res Commun* 1991;179:1361-1367.
- 9 Song K, Kurobe Y, Kanehara H, Wada T, Inada Y, Nishikawa K, Miyazaki M: Mapping of angiotensin II receptor subtypes in peripheral tissues of spontaneously hypertensive rats by in vitro autoradiography. *Clin Exp Pharmacol Physiol* 1995;22:S17-S19.
- 10 Nora EH, Munzenmaier DH, Hansen-Smith FM, Lombard JH, Greene AS: Localization of the ANG II type 2 receptor in the microcirculation of skeletal muscle. *Am J Physiol* 1998;275:H1395-H1403.
- 11 Lombard JH, Sylvester FA, Phillips SA, Frisbee JC: High-salt diet impairs vascular relaxation mechanisms in rat middle cerebral arteries. *Am J Physiol Heart Circ Physiol* 2003;284:H1124-H1133.
- 12 Weber DS, Lombard JH: Elevated salt intake impairs dilation of skeletal muscle resistance arteries via angiotensin II suppression. *Am J Physiol Heart Circ Physiol* 2000;278:H500-H506.
- 13 Weber DS, Lombard JH: Angiotensin II AT1 receptors preserve vasodilator reactivity in skeletal muscle resistance arteries. *Am J Physiol Heart Circ Physiol* 2001;280:H2196-H2202.
- 14 Auch-Schwelk W, Katusic ZS, Vanhoutte PM: Thromboxane A2 receptor antagonist inhibit endothelium dependent contractions. *Hypertension* 1990;15:699-703.
- 15 Drenjancevic-Peric I, Lombard JH: Reduced Angiotensin II and Oxidative Stress Contribute to Impaired Vasodilation in Dahl Salt-Sensitive Rats on Low-Salt Diet. *Hypertension* 2005;45:687-691.
- 16 Phillips SA, Drenjancevic-Peric I, Frisbee JC, Lombard JH: Chronic AT1 receptor blockade alters mechanisms mediating responses to hypoxia in rat skeletal muscle resistance arteries. *Am J Physiol* 2004;287:H545-H552.
- 17 Phillips SA, Lombard JH: Chronic AT1 receptor blockade alters the mechanisms mediating hypoxic dilation in middle cerebral arteries. *J Cardiovasc Pharmacol* 2005;46:706-712.
- 18 Hornig B, Landmesser U, Kohler C, Ahlersmann D, Spiekermann S, Christoph A, Tatge H, Drexler H: Comparative effect of ace inhibition and angiotensin II type I receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase. *Circulation* 2001;103:799-805.
- 19 Prasad A, Tupas-Habib T, Schenke WH, Mincemoyer R, Panza JA, Wacławin MA, Ellahham S, Quyyumi AA: Acute and chronic angiotensin-1 receptor antagonism reverses endothelial dysfunction in atherosclerosis. *Circulation* 2000;101:2349-2354.
- 20 Amaral SL, Papanek PE, Greene AS: Angiotensin II and VEGF are involved in angiogenesis induced by short-term exercise training. *Am J Physiol Heart Circ Physiol* 2001;281:H1163-H1169.
- 21 Frisbee JC, Lombard JH: Short-term angiotensin converting enzyme inhibition reduces basal tone and dilator reactivity in skeletal muscle arterioles. *Am J Hypertens* 2000;13:389-395.
- 22 Huxley VH: Sex and the cardiovascular system: the intriguing tale of how women and men regulate cardiovascular function differently. *Advan in Physiol Edu* 2007;31:17-22.
- 23 Miller VM: Sex-based differences in vascular function. *Women's Health* 2010;6:737-752.
- 24 Ruzic A, Miletic B, Nola AI, Persic V, Razov Radas M, Vcev A: Endotelna disfunkcija u enigmatskoj slagalici kardiovaskularnih bolesti. *Med glas* 2009;6:2-15.
- 25 Crakowski JL, Minson CT, Salvat-Melis M, Halliwill JR: Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends Pharmacol Sci* 2006;9:503-509.
- 26 Wright CI, Kroner CI, Draijer R: Non-invasive methods and stimuli for evaluating the skin's microcirculation. *J Pharmacol Toxicol Methods* 2006;54:1-25.
- 27 Koller A, Bagi Z: On the role of mechanosensitive mechanisms eliciting reactive hyperemia. *Am J Physiol Heart Circ Physiol* 2002;283:H2250-H2259.
- 28 Cattella F, Healy D, Lawson JA, Fitzgerald GA: 11-Dehydrothromboxane B2: a quantitative index of thromboxane A2 formation in the human circulation. *PNAS* 1986;83:5861-5865.
- 29 Reed R, Kolz C, Potter B, Rocic P: The mechanistic basis for the disparate effects of angiotensin II on coronary collateral growth. *Arterioscler Thromb Vasc Biol* 2008;28:61-67.

- 30 Sacks F, Svetkey L, Vollmer W, Appel L, Bray G, Harsha D et al. Effects on blood pressure of reduced dietary sodium and the dietary approaches to stop hypertension (DASH) diet. *N Engl J Med* 2001;344:3-10.
- 31 Naruse M, Tanabe A, Sato A, Takagi S, Tsuchiya K, Imaki T, Takano K: Aldosterone Breakthrough During Angiotensin II Receptor Antagonist Therapy in Stroke-Prone Spontaneously Hypertensive Rats. *Hypertension* 2002;40:28-33.
- 32 Verhave JC, Hillege HL, Burgerhof JG, Janssen WM, Gansevoort RT, Navis GJ, de Zeeuw D, de Jong PE: Sodium intake affects urinary albumin excretion in overweight subjects. *J Intern Med* 2004;256:324-330.
- 33 Swift PA, Markandu ND, Sagnella GA, He FJ, Macgregor GA: Modest salt reduction reduces blood pressure and urine protein excretion in black hypertensives. A randomized control trial. *Hypertension* 2005;46:308-312.
- 34 Heeg JE, de Jong PE, van der Hem GK, de Zeeuw D: Efficacy and variability of the antiproteinuric effect of ACE inhibition by lisinopril. *Kidney Int* 1989;36:272-279.
- 35 Hernandez I, Cowley AW Jr, Lombard JH, Greene AS: Salt intake and angiotensin II alter microvessel density in the cremaster muscle of normal rats. *Am J Physiol Heart Circ Physiol* 1992;263:H664-H667.
- 36 Lombard JH, Bizub D, Weber DS, Greene AS: Acute elevation of salt intake impairs electrical and mechanical responses of rat middle cerebral arteries to reduced PO₂ and iloprost via suppression of angiotensin II (Abstract). *Microcirculation* 1997;5:159.
- 37 Munzenmaier DH, Greene AS: Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension* 1996;27:760-765.
- 38 Nurkiewicz TR, Boegehold MA: Reinforcement of arteriolar myogenic activity by endogenous ANG II: susceptibility to dietary salt. *Am J Physiol Heart Circ Physiol* 2000;279:H269-H278.
- 39 Frisbee JC, Weber DS, Lombard JH: Chronic captopril administration decreases vasodilator responses in skeletal muscle arterioles. *Am J Hypertens* 1999;12:705-715.
- 40 Davidge ST: Prostaglandin H synthase and vascular function. *Circ Res* 2001;89:650-660.
- 41 Mazzolai L, Maillard M, Rossat J, Nussberger J, Brunner HR, Burnier M: Angiotensin II receptor blockade in normotensive subjects: a direct comparison of three AT1 receptor antagonists. *Hypertension* 1999;33:850-855.
- 42 Maeso R, Navarro-Cid J, Munoz-Garcia R, Rodrigo E, Ruilope LM, Lahera V, Cachofeiro V: Losartan reduces phenylephrine constrictor response in aortic rings from spontaneously hypertensive rats: role of nitric oxide and angiotensin II type 2 receptors. *Hypertension* 1996;28:967-972.
- 43 Muller C, Endlich K, Barthelmebs M, Helwig JJ: AT2-antagonist sensitive potentiation of angiotensin II-induced vasoconstrictions by blockade of nitric oxide synthesis in rat renal vasculature. *Br J Pharmacol* 1997;122:1495-1501.
- 44 Tsutsumi Y, Matsubara H, Masaki H, Kurifara K, Marusawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawanda N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T: Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest* 1999;104:925-935.
- 45 Barber MN, Sampey DB, Widdop RE: AT2 receptor stimulation enhances antihypertensive effect of AT1 receptor antagonist in hypertensive rats. *Hypertension* 1999;34:1112-1116.
- 46 Henrion D, Kubis N, Levy BI: Physiological and Pathophysiological Functions of the AT2 Subtype Receptor of Angiotensin II: From Large Arteries to the Microcirculation. *Hypertension* 2001;38:1150-1157.
- 47 Matrougui K, Levy BI, Henrion D: Tissue angiotensin II and endothelin-1 modulate differently the response to flow in mesenteric resistance arteries of normotensive and spontaneously hypertensive rats. *Br J Pharmacol* 2000;130:521-526.
- 48 Usui M, Egashira K, Tomita H, Koyanagi M, Katoh M, Shimokawa H, Takeya M, Yoshimura T, Matsushima K, Takeshita A: Important role of local angiotensin II activity mediated via type 1 receptor in the pathogenesis of cardiovascular inflammatory changes induced by chronic blockade of nitric oxide synthesis in rats. *Circulation* 2000;101:305-310.
- 49 Munoz-Garcia R, Navarro-Cid J, Ruilope LM, Lahera V, Cachofeiro V: Losartan reduces constrictor responses to endothelin-1 and the thromboxane A2 analogue in aortic rings from spontaneously hypertensive rats: role of nitric oxide. *J Hypertens* 1997;15:1677-1684.
- 50 Bertolino F, Valentin JP, Maffre M, Jover B, Bessac AM, John GW: Prevention of thromboxane A2 receptor-mediated pulmonary hypertension by a nonpeptide angiotensin II type 1 receptor antagonist. *J Pharmacol Exp Ther* 1994;268:747-752.
- 51 Valentin JP, Jover B, Maffre M, Bertolino F, Bessac AM, John GW: Losartan prevents thromboxane A2/prostanoid (TP) receptor mediated increase in microvascular permeability in the rat. *Am J Hypertens* 1997;10:1058-1063.