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Sex and salt intake dependent renin-angiotensin plasticity in the liver of the rat

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Objective. Epidemiological studies confirm that hypertensive patients respond differently to renin-angiotensin system (RAS) inhibition depending on their gender. The aim of present work is to focus on sex-dependent differences in RAS regulation under conditions of increased salt intake.

Method. To investigate RAS, we measured the expression of angiotensinogen (Agt) mRNA, angiotensin receptor type 1 (AT1) mRNA and mitochondria assembly receptor (MasR) in the liver of rats under control conditions and after feeding with a salt diet (2% NaCl). In parallel, vascular endothelial growth factor A (VEGF-A) mRNA was analyzed.

Results. Regression analysis revealed sex-dependent differences in the correlation between mRNA expression of AT1 and that of Agt, MasR and VEGF-A in both groups. There was a significant negative correlation between AT1 and Agt mRNA expression in the male control group, but this correlation disappeared in males exposed to a salt diet. In females, AT1 and Agt expression correlated only in the group exposed to the salt diet. In control males, there was a borderline trend to correlation between AT1 and MasR mRNA expression. The correlation between AT1 and VEGF-A mRNA expression was significant only in the control females, however, after exposure to a salt diet, this correlation diminished.

Conclusions. We hypothesize that RAS components expression is compensated differently in males and females. The observed loss of compensatory relationships in RAS between AT1 and Agt and AT1 and MasR in male rats under a salt diet can contribute to the differences observed in human with hypertension associated with an unhealthy diet.

Key words: hypertension, salt diet, male, female, AT1 receptor, angiotensinogen, Mas receptor, VEGF-1

In 2015, cardiovascular diseases (CVDs) caused nearly one third of all deaths in Europe (Townsend et al. 2016). Hypertension is one of the most important risk factors for the development of other CVD (Shapo et al. 2003). Many epidemiological studies have revealed risk factors that are associated with an increased blood pressure. Some of these factors include age, obesity, dyslipidemia, and an unhealthy lifestyle (increased dietary salt intake, alcohol consumption, smoking, etc.). All of these studies also revealed significant sex-dependent differences in the prevalence of hypertension (Shapo et al. 2003; Bener et al. 2004; Wei et al. 2015). Some epidemiological studies have reported that incidence of hypertension is lower in premenopausal women than in age-matched men, however, after menopause, this difference disappears and the incidence of hypertension starts to rise in women (Nelson and Coady,

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2008; Sandberg and Ji 2012; Mozaffarian et al. 2016). Blood pressure is higher in women after menopause than in premenopausal women (Staessen et al. 1989). In Europe, there were more death incidentes from cardiovascular diseases in men than in women before 65 years of age in 2015 (Townsend et al. 2016).

The renin-angiotensin system (RAS) plays a key role in the regulation of blood pressure and homeostasis of electrolytes in the body fluids. Many drugs used in the widespread treatment of hypertension are based on targeting of RAS components (Steckelings et al. 2011).

The liver is the primary source of angiotensinogen (Agt) for central RAS in the circulation, and other components of the RAS are also expressed in the liver (Lubel et al. 2009; Matsusaka et al. 2012). The main effector molecule of the classical axis of RAS is angiotensin II (AngII). AngII is synthesized from angiotensinogen by a two-step enzymatic cascade. Agt is converted by renin to angiotensin I (AngI), and AngI is cleaved by angiotensin converting enzyme (ACE) to AngII in the following step (Reid 1998). Through the AT1 receptor, AngII mediates vasoconstriction and fibrosis progression and induces the proliferation of hepatocytes in the liver. AngII also mediates some key aspects of liver tissue repair – myofibroblast proliferation, the infiltration of inflammatory cells and collagen synthesis (Yoshiji et al. 2001; Bataller et al. 2005).

Recent studies have revealed that AngII metabolite angiotensin (1–7) [Ang (1–7)] also plays a role in the regulation of blood pressure. Ang (1–7) is a part of the so-called second axis of RAS that shows antagonistic effects against the classical first axis (Iwai and Horiuchi 2009; Slamkova et al. 2016). Ang (1–7) acts via the Mas receptor (mitochondria assembly receptor) (Santos et al. 2003) to induce vasodilatation and inhibits tissue remodeling and fibrosis in the liver (Lubel et al. 2009).

The higher intake of NaCl can affect the blood pressure and negative consecutive effects can be reversed by the suppression of RAS activity (He and MacGregor 2003; Cholewa et al. 2005). However, the treatment of hypertension is complicated by regulatory feedback connections between RAS components. The inhibition of AngII formation leads to an increased expression of renin in the kidney (Kammerl et al. 2002). Increased levels of AngII resulted in a significant decrease in angiotensin receptor binding in the rat mesenteric artery (Gunther et al. 1980). After ACE activity blockade by ACE inhibitors, AngII can still be produced by other enzymes, mainly by chymase. These ACE-independent pathways may contribute to the restoration of AngII activity through the AT1 receptor or increase the stimulation of the second axis of RAS (Nehme and Zibara 2017).

Local tissue RAS in the liver is mainly associated with regeneration processes after injury, fibrosis progression or cell proliferation and apoptosis in healthy and malignant tissue (Koh et al. 2010; Moreira de Macedo et al. 2014; Simoes E Silva et al. 2017). On the other hand, very little is known about role of liver RAS in the regulation of hypertension. In recent studies an attention was mainly focused on the analyses of RAS components expression in the kidneys (Rands et al. 2012; Mao et al. 2013).

Therefore, our work is focused on the analysis of the reciprocal interactions of RAS in the liver in a higher salt diet model in males and females.

Materials and methods

The experimental protocol was approved by the Ethical Committee for the Care and Use of Laboratory Animals at the Comenius University in Bratislava and the State Veterinary and Food Administration of the Slovak Republic Committee of Slovak Republic. The investigation conditions were in accordance with the guidelines with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Animals. Parental animals (Velaz, Prague, Czech Republic) were housed in a temperature-controlled room (21±2 °C) under a 12 h:12 h light:dark regime with lights on at 7:00 h. After distinguishing the phase of the ovulation cycle, females were mated with males. Mating was confirmed by the detection of sperm in a vaginal smear. After that females were transferred to the separate cages. During the gravidity, the females were fed with standard laboratory chow. After birth, litters were culled to four females and four males per dam. All offspring were fed a diet with normal salt content (0.5% NaCl) *ad libitum*. Systolic blood pressure (SBP) was measured regularly from the 10th week until the end of the experiment. At the 14th week of the age, the animals of both sexes were split into two groups. The first group was fed with standard laboratory chow (0.5% NaCl) *ad libitum* and served as a control group, while the second group was fed a higher salt content chow (2% NaCl) *ad libitum* during the last three weeks of the experiment (weeks 15–17). Sampling was performed at the 17th of the age. Livers were removed and stored at –80 °C until RNA isolation was performed. The

heart and the left ventricle were weighed. Relative heart weight (RHW) and relative left ventricle weight (RVW) were calculated as a ratio to the body weight (BW).

RNA isolation and real-time PCR. Total RNA from the liver (70 mg of tissue) was isolated with the use of RNAzol (MRC, USA). The synthesis of cDNA was carried out with the use of kit ImProm-II Reverse Transcription System II (Promega, USA) according to the manufacturer's instructions. The quantification of cDNA was performed by real-time PCR using the QuantiTect SYBR Green PCR kit (Qiagen, Germany) and the StepOne™ Plus Real-Time PCR System thermocycler (Applied Biosystems, USA). The primers used for amplification were: *Agt* (AH003514.1) sense 5'-CTC AGG CCA AGC TGT CTA CC-3', antisense 5'-CGT AGA TGG CGA ACA GGA AC-3'; *at1r* (NM_030985) sense 5'-CCA AGA TGA CTG CCC CAA G-3', antisense 5'-ATC ACC ACC AAG CTG TTT CC-3'; *MasR* (NM_012757.2) sense 5'-CCA GAG AGA AAA TGG CCT GAA G-3', antisense 5'-TCC TCA TCC GGA AGC AAA GG-3'; and *vegf-a* (NM_031836.3) sense 5′-GCA GCG ACA AGG CAG ACT AT-3', antisense 5′-GCA ACC TCT CCA AAC CGT TG-3'. Real-time PCR conditions were: hot-start at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 49–55 °C for 30 s and 72 °C for 30 s. The specificity of the PCR reaction was validated by melting curve analysis.

Statistical analyses. Correlations in the expression of mRNA associated with RAS were evaluated by correlation analysis. Sex differences in BW, SBP, RHW, and RVW were analyzed by two-way ANOVA followed by post-hoc Tukey test or by regression analysis.

Table 1
Body weight, relative heart weight, relative left ventricle weight and systolic blood pressure of rats at the end of experiment in males and females with (2% NaCl) or without (Control) higher salt diet.

Parameter	Group	Sex	Mean	SEM	p-value
BW	Control	Males	471.7	± 12.68	a
		Females	267.3	± 6.89	b
	2% NaCl	Males	450.2	± 9.50	a
		Females	267.8	± 7.39	b
RHW	Control	Males	2.2	± 0.07	a
		Females	2.6	± 0.06	b
	2% NaCl	Males	2.2	± 0.02	a
		Females	2.6	± 0.04	b
RVW	Control	Males	1.2	± 0.06	a
		Females	1.5	± 0.08	b
	2% NaCl	Males	1.2	± 0.03	a
		Females	1.4	± 0.04	b
SBP	Control	Males	130.0	± 2.97	a
		Females	121.0	± 33.47	a
	2% NaCl	Males	134.0	± 3.86	a
		Females	124.4	± 4.89	a

Results from each physiological parameter were analyzed by two-way ANOVA (sex and salt diet) followed by post-hoc Tukey test. Values with the same letter are not significantly different. Data are given as a mean ± SEM (n=6–7). Abbreviations: BW – body weight; RHW – relative heart weight; RVW – relative ventricle weight; SBP – systolic blood pressure.

Results

According two-way ANOVA salt diet treatment did not influence the systolic blood pressure, body weight, RHW, and RVW in males or females, however, males showed higher body weight (ANOVA, $p<0.001$) and lower RHW (ANOVA, $p<0.001$) and RVW (ANOVA, $p<0.001$) compared to females in both, control as well as salt diet exposed animals (Table 1). SBP showed sex dependent difference with increased levels observed in males compared to females (ANOVA; $p<0.05$). However, post hoc analysis did not show differences between control and salt diet treated subgroups (Table 1). Systolic blood pressure was increasing in males, but not females (data not shown), during the experiment in the control group (Figure 1; $y=1.6164x+99.234$; $R=0.425$; $p<0.01$) and also in the group provided with the salt diet (Figure 1; $y=1.5967x+105.33$; $R=0.373$; $P<0.01$).

Sex differences in the correlation between AT1 and Agt mRNA expression. Higher salt content in the food induced changes in the correlation between AT1 and Agt mRNA expression in a sex-specific manner. The higher salt diet was associated with a significant positive correlation between AT1 and Agt mRNA expression in female rats (Figure 2B; $y=0.9483x+77.545$; $R=0.915$; $p<0.01$). We did not observe this correlation in female rats kept under the control diet (Figure 2A). On the other hand, in control males, we observed a significant negative correlation between AT1 and Agt (Figure 2C; $y=-0.8905x+1841.6$; $R=0.858$; $p<0.05$). In males consuming the higher salt diet, this correlation diminished (Figure 2D).

Sex differences in the correlation between AT1 and VEGF-A mRNA expression. We observed a significant positive correlation between AT1 and VEGF-A mRNA expression in control female rats (Figure 3A; $y = 0.3477x + 393.41$; $R=0.856$; $p<0.05$). In the female group exposed to the higher salt diet, this correlation diminished (Figure 3B).

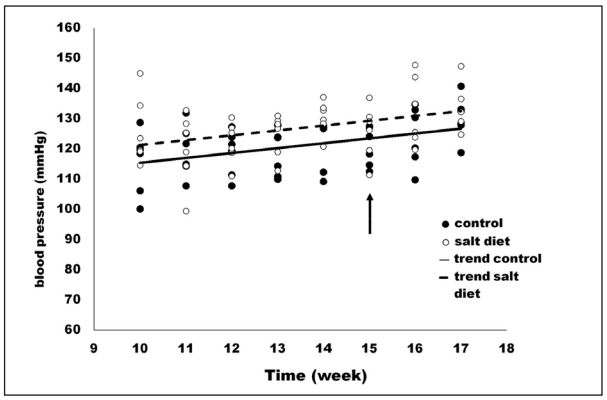

Figure 1. Systolic blood pressure in males in the control group (●) and the group fed with the higher salt diet (○). The black arrow demonstrates when the feeding with the higher salt diet began. The x-axis shows the age of animals. The solid line demonstrates a significant linear trend in control males, while the broken line demonstrates a significant linear trend in males fed with the higher salt diet.

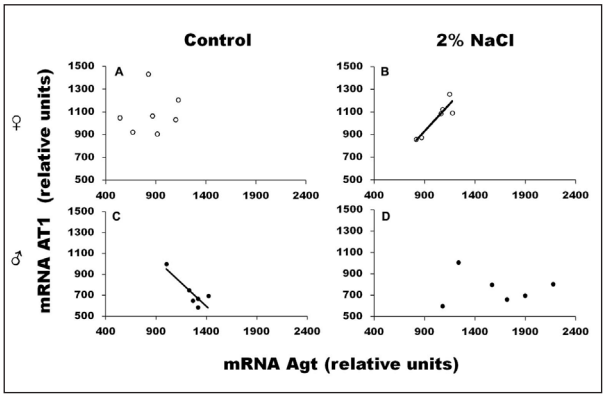

Figure 2. The correlation between AT1 and Agt mRNA expression in the liver of female (○) (**A, B**) and male (●) (**C, D**) rats after feeding with the control (**A, C**) or higher salt diets (**B, D**). The solid line demonstrates a significant correlation ($p<0.05$; n=6–7). Abbreviations: AT1 – angiotensin receptor type 1; Agt – angiotensinogen.

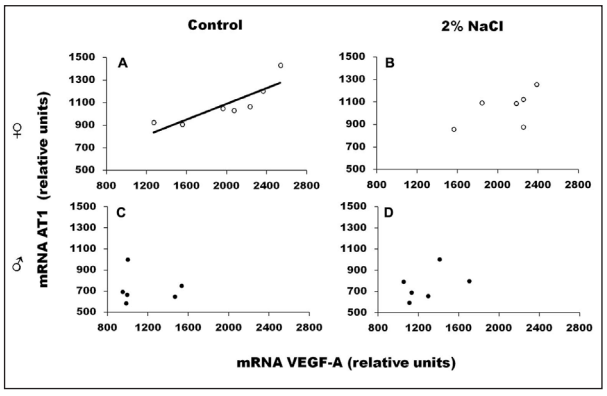

Figure 3. The correlation between AT1 and VEGF-A mRNA expression in the liver of female (○) (**A, B**) and male (●) (**C, D**) rats after feeding with the control (**A, C**) or higher salt diets (**B, D**). The solid line demonstrates a significant correlation ($p<0.05$; n=6–7). Abbreviations: AT1 – angiotensin receptor type 1; VEGF-A – vascular endothelial growth factor A.

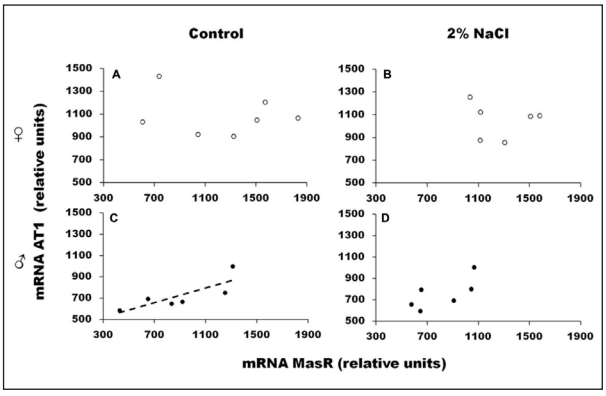

Figure 4. The correlation between AT1 and MasR mRNA expression in the liver of female (○) (**A, B**) and male (●) (**C, D**) rats after feeding with the control (**A, C**) or higher salt diets (**B, D**). The broken line demonstrates a borderline non-significant trend ($p=0.051$; n=6–7). Abbreviations: AT1 – angiotensin receptor type 1; MasR – mitochondria assembling receptor.

Sex differences in the correlation between AT1 and MasR mRNA expression. Correlation analysis revealed a borderline trend towards a correlation between AT1 and MasR mRNA expression in control male rats (Figure 4C; y=0.3435x+417.31; R=0.805; p=0.051), but not in female rats (Figure 4A, B). In male rats consuming higher salt diet, this trend diminished (Figure 4D; y=0.4739x+373.77; R=0.712; p=0.112).

Discussion

Compensatory relationships inside the renin-angiotensin system at the level of Agt, AT1, and MasR mRNA expression were studied and related to VEGF-A mRNA levels in the liver of female and male rats. Correlation analyses revealed sex-dependent differences in response to the higher salt diet (Figure 5).

In males, we observed a negative correlation between AT1 and Agt mRNA expression in the control group. It is known that high Agt expression results in an increase in AngII levels (Kobori et al. 2001; Singh et al. 2003). Therefore, we hypothesize that increased Agt expression can (at least to some extent) also indicate an increase in AngII concentration. Several experimental evidences showed that AngII administration influenced AT1 expression. The treatment of the cells with AngII (in a model of BAC – Bovine adrenal fasciculata cells and PC12W

cells – rat pheochromocytoma PC12 cell line) resulted in a decrease of AT1 receptor mRNA expression (Ouali et al. 1997). Rat cardiomyocytes exposed to AngII showed reduced AT1 receptor mRNA levels. The maximal decrease was observed after 6 hours of exposure. Then, the expression of the AT1 receptor started to restore to original levels (Chen et al. 2002). Increased levels of AngII resulted in a significant reduction of its binding on angiotensin receptors in the rat mesenteric artery (Gunther et al. 1980). Our previous study has revealed that chronic AngII infusion is associated with a trend towards the down-regulation of AT1 expression in rat hearts compared to the control group (Herichova et al. 2013). All of the above-mentioned findings are in accordance with the recently presented negative correlation between Agt and AT1 mRNA expression. However, in some studies, this negative feedback was not observed. Treatment of the mouse neuronal cell line with AngII led to the increase in AT1 receptor expression (Mitra et al. 2010). In Sprague-Dawley male rats, chronic AngII infusion (13 days) did not change the expression of the AT1 receptor in the liver and kidney but increased the expression of AT1 mRNA in the adrenal gland (Harrison-Bernard et al. 1999).

Uncertainty about the effect of AngII on AT1 expression was explained by Schiffrin et al. (1983), who have described the bimodal interactions between AngII and the AT1 receptor. Treatment with lower doses of AngII leads to the suppression of AT1 receptor number, while high doses of AngII stimulate AT1 expression *in vivo* via an aldosterone-dependent mechanism. We suppose that the increase in AngII levels was rather low in our study and propose that this could generate a negative feedback for AT1 mRNA expression in the male control group.

In the male control group, we also observed a borderline significant correlation between AT1 and Mas receptor mRNA expression in the liver.

The Mas receptor and AT1 receptor belong to the family of G-coupled receptors (Reid 1998; Santos et al. 2003). The Mas and AT1 receptors can form both homodimers and heterodimers, with dimerization leading to crosstalk and the modulation of receptor activity (Lyngso et al. 2009). The co-expression of AT1 and Mas receptor in CHO-K1 cells led to a decrease in AT1 receptor binding. On the other hand, Mas and AT1 co-expression was associated with an enhanced cell-surface expression of AT1 receptor (Kostenis et al. 2005). This experiment implies that the positive correlation between Mas and the AT1 receptor mRNA expression observed in our study works as a negative regulation pathway for AT1 receptor activity.

Both of the above-described correlations observed in the male control group were not observed in higher salt-treated males. It seems that the higher salt diet causes a disturbance in regulatory relationships inside the RAS. Similarly, we did not observe these compensation mechanisms in the female control group.

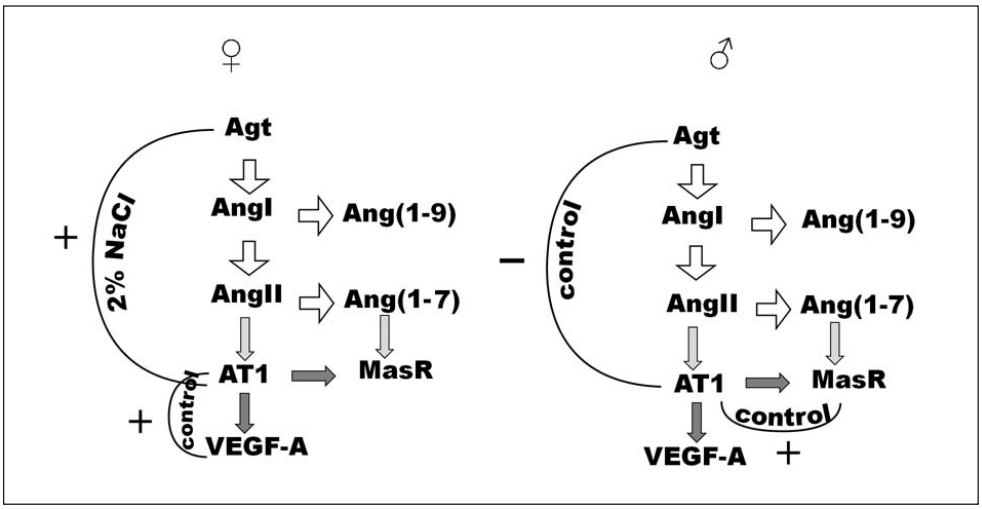

Figure 5. Gender differences in the expression of RAS components in the liver of rats consuming normal or higher salt (2% NaCl) diets. The arch denotes a positive (+) or negative (–) correlation between RAS components under the conditions of control (control) or higher salt (2% NaCl) diets. The white arrows indicate the progression of the AngII biosynthetic pathway, light grey arrows show the interaction with receptors and the dark grey arrows denote regulatory pathways. Abbreviations: Agt – angiotensinogen; AngI – angiotensin I; AngII – angiotensin II; Ang (1–7) – angiotensin (1–7); Ang (1–9) – angiotensin (1–9); AT1 – angiotensin receptor type 1; MasR – mitochondria assembly receptor; VEGF-A – vascular endothelial growth factor A.

RAS components are also involved in angiogenesis, mainly by regulation of the expression of growth factors like VEGF-A (Imai et al. 2007). AngII, acting via the AT1 receptor, increased the expression of VEGF-A mRNA in human liver cancer cells (Fan et al. 2016). In animals treated with AngII infusion, the expression of VEGF-A (both protein and mRNA) in the retina increased. This effect was reversed after the administration of AT1 or AT2 antagonist (Zhang et al. 2004).

Angiogenesis mediated by VEGF-A is one of the key factors associated with the progression of cancer (Yoshiji et al. 2002; Ye et al. 2015; Zhao and Adjei 2015; Taurone et al. 2016). Numerous studies have revealed that the treatment of hypertension, targeted to RAS components (AT1 blockers, ACE inhibitors), is associated with the suppression of VEGF-A-mediated tumor development, better survival and a lower risk of the development of cancer (Yoshiji et al. 2002; Dai et al. 2015; Fan et al. 2016). Moreover, a recent study implied that breast cancer cells increased the expression of VEGF-A after exposure to high NaCl levels compared to control cells (Amara et al. 2016). In our experiment, we observed a significant positive correlation between the AT1 receptor and VEGF-A mRNA expression in the liver of the female control group. In the females with a higher salt intake, this correlation diminished.

We also analyzed the association of higher salt intake with changes in blood pressure and weight. Blood pressure in males gradually increased during the experiment, but this increase was independent of salt intake. In our study, the higher salt diet did not influence body weight, heart weight, left ventricle weight or systolic blood pressure in either sex.

Previously, it has been demonstrated that feeding with an 8% high salt diet led to a decrease in body weight and an increase in heart weight compared to the control group. Animals fed with a high salt diet also showed a significantly higher systolic and diastolic blood pressure compared to the control group (Ogihara et al. 2001; Hayakawa et al. 2015). The HW/BW ratio was significantly higher in the high salt group compared to the control group (Hayakawa et al. 2015; Igreja et al. 2019). We hypothesize that the above-mentioned studies referred to a more pronounced effect of the salt diet because they used a higher dose of salt (8%) than our study (2%), where changes induced by the salt diet were most probably compensated for by the homeostatic apparatus of the body. Another important factor may be the duration of the experiment. High salt intake led to much more pronounced changes in blood pressure after eight weeks of treatment compared to the second week of the experiment (Ogihara et al. 2001).

We revealed several interactions between RAS components and VEGF-A expression in the liver. These interactions were sex-dependent. Our findings are in accordance with previous research focused on gender-differences in RAS activity. Men and women respond differently to RAS pharmacological modulation. After treatment with lisinopril (ACE inhibitor), there was a greater decrease of blood pressure in men than in women (Falconnet et al. 2004). Women with congestive heart failure treated with angiotensin receptor blockers had better survival than women treated with ACE inhibitors (Hudson et al. 2007). Os et al. (1994) have revealed that a cough was reported three times more frequently in women than in men as a side effect of ACE inhibitors. It was shown that AT1 receptor binding is significantly higher in the kidney glomeruli of male rats compared to females (Rogers et al. 2007). A study of a cohort of patients from the Swedish Primary Care Cardiovascular Database (SPCCD) has revealed that there are also sex differences in antihypertensive drug prescription. Women were more frequently treated with diuretics and beta blockers than males, while ACE inhibitors and calcium channel blockers were more often prescribed to men. This discrepancy was explained by the different frequency of some side effects of treatments in men and women (Ljungman et al. 2014).

Sex-dependent differences in RAS treatment response can be associated with levels of sex-hormones in men and women. After treatment with 2-methoxyestradiol (2ME2), a significant down-regulation of AngII binding to the AT1 receptor was observed in rat liver epithelial cells. Expression of the AT1 receptor in cells was also suppressed after treatment with 2ME2 (Koganti et al. 2012).

All of these studies point to the important role of sex-dependent factors in the development and treatment of hypertension and related diseases that can be at least partly caused by differences in the internal RAS regulatory feed-back relationships.

To conclude, the main aim of this study was to analyze the regulatory feedback interactions inside RAS and their associations with VEGF-A mRNA expression in the liver. The observed interactions were sex-dependent and were modified after exposure to a higher salt diet. Compensatory correlations between AT1 and Agt, and AT1 and Mas receptors observed in males disappeared after exposure to a higher salt diet. The positive correlation between AT1 and VEGF-A observed in females also disappeared

after the administration of a diet with 2% NaCl. We believe that our study may contribute to a better understanding of sex differences in the development of hypertension induced by a higher salt intake.

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