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Gender Differences in Renin Angiotensin Aldosterone System Affect Extra Cellular Volume in Healthy Subjects.

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ABSTRACT

OBJECTIVE Several studies reported gender differences in aldosterone. It is unknown whether these differences are associated with differences in volume regulation. Therefore, we studied both aldosterone and extracellular volume in men and women on different sodium intakes. **METHODS** In healthy normotensive men (n=18) and premenopausal women (n=18) we investigated plasma aldosterone, blood pressure, and extracellular volume (^{125}I-iothalamate), during both a low (target intake 50 mmol Na^+/day) and high sodium intake (target intake 200 mmol Na^+/day) in a cross-over set-up. Furthermore, we studied the adrenal response to angiotensin II infusion (0.3, 1.0 and 3.0 ng/kg/min for 1 h) on both sodium intakes. **RESULTS** Men had a significantly higher plasma aldosterone, extracellular volume and systolic blood pressure than women during a high sodium intake ($p<0.05$). During a low sodium intake, extracellular volume and blood pressure were higher in men as well ($p<0.05$), whereas the difference in plasma aldosterone was no longer significant ($P=0.252$). The adrenal response to exogenous angiotensin II was significantly lower in men than in women on both sodium intakes. **CONCLUSIONS** Constitutive gender differences in the regulation of aldosterone, characterized by a higher aldosterone and a lower adrenal response to exogenous angiotensin II infusion in men, are associated with a higher extracellular volume and blood pressure in men. These findings suggest that gender differences in the regulation of aldosterone contribute to differences in volume regulation between men and women.

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49 **Key words**: Extracellular volume, Renin-Angiotensin System, Gender,

50 Aldosterone, Healthy Volunteers.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) is a main regulatory system of volume homeostasis and blood pressure. Aldosterone secretion induces sodium and water retention in the distal tubules of the kidneys, and is stimulated by angiotensin II (ang II) and a high plasma potassium concentration.

Differences in the RAAS between men and women have been described(8, 10, 14, 17, 18). Higher aldosterone levels have been reported in men, both in normotensive and in hypertensive subjects(9, 17). However, it is unknown whether gender differences in aldosterone levels are associated with functional consequences on volume homeostasis(14). As the major effect of aldosterone is sodium and water retention, we hypothesize that a higher aldosterone level in men is associated with a higher extracellular volume (ECV). Furthermore, gender differences in regulation of aldosterone production are not well studied.

To study gender differences in RAAS and ECV, maintaining standardized study conditions is mandatory. RAAS hormone levels vary with sodium diet and, in women, with phase of the menstrual cycle(24). Therefore, in this study we investigated gender differences in aldosterone levels, ECV and blood pressure during a low and high sodium intake, in a steady state and standardized for menstrual cycle. Furthermore, we studied gender differences in the adrenal response to ang II infusion in these standardized conditions, during both sodium intakes.

METHODS

Study population

The study population consisted of 36 healthy, Caucasian subjects (women, n=18; men, n=18) which took part in the GRECO program, which is an ongoing study program on renal hemodynamic studies in different populations (healthy and chronic kidney disease patients) with standardized measurements and harmonized protocols for different subsequent studies, allowing combined analyses of the different sub-studies. The women were studied in the RETAP sub-study and compared with men from the Gene-Environment sub study (15, 29). All subjects were non-smokers and normotensive, having a sitting systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg measured by Dinamap, and were not treated with an antihypertensive drug. Their medical history revealed no significant diseases. Subjects with obesity (BMI > 30 kg/m^2 at screening) were excluded. Physical examination and electrocardiography did not reveal any abnormalities. None of the women were users of oral contraceptive medication, or were pregnant. Both studies were approved by the local medical ethical committee (METc number: RETAP study 2010/294, www.trialregister.nl; trial registration number: 2635, Gene-Environment study 2001/012) and all subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study protocol

In both women and men, a standardized cross-over protocol was performed as described earlier(26, 29), which consisted of two one-week periods: in random order a 7-day period on a low sodium diet (LS; aim: 50 mmol Na^+/day), and a 7-day period on a high sodium diet (HS; aim: 200 mmol Na^+/day), with a stable potassium intake. This was achieved by dietary counseling. For assessment of dietary compliance and the achievement of a stable sodium balance, 24h urine was collected at day 3 and day 6 during each period. In men the study periods were done consecutively, and in women these periods were divided by one menstrual cycle, to avoid the influence of momentarily sex hormones to aldosterone levels and ang II responsiveness(14, 19). At day 7 of both study periods, during which all women were in the mid-follicular phase (day 7±2 of menstrual cycle), the subjects reported at the research unit at 8am after an overnight fast. Body weight, length and waist-to-hip ratio were measured at the start of this day. An intravenous cannula was inserted into each forearm, one for drawing blood samples, the other for infusion of ang II. Subjects received standardized meals and fluids during the day, with sodium intake adjusted to the prescribed diet. To ensure sufficient urine output, infusion of 250 mL/h of 5% glucose was administered and every hour 250 mL of oral fluids were provided. Baseline values for blood pressure were obtained from 10am to 12am. Between 12am and 3pm ang II (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered intravenously, at a constant rate in doses of 0.3, 1 and 3 ng/kg/min each during 1h.

Blood pressure and heart rate were measured with an automated sphygmomanometer (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA) at 15-min intervals. Subjects were seated in a quiet room in a semi-supine position, with their arm in resting position. During ang II infusions, blood pressure was measured at 5-min intervals. Appropriate blood pressure cuff was determined on the basis of arm circumference.

ECV was measured as the distribution volume of ^{125}I-iothalamate during steady state, as described in more detail previously(30). This was performed before ang II infusions. Briefly, the distribution volume of ^{125}I-iothalamate is calculated from the plasma level of ^{125}I-iothalamate divided by the total amount of ^{125}I-iothalamate in the body, which equals the amount infused minus the amount excreted. It is calculated as sum(I × V) + Bolus − sum(U × V)/P, and expressed as ECV/body surface area (BSA), i.e., l/1.73 m^2 BSA. BSA was calculated according to the DuBois-DuBois formula(7).

Sample collection and analytical methods

Blood samples were drawn at baseline and after each hour of ang II infusion. Blood for measuring plasma aldosterone and renin was collected in precooled tubes and immediately centrifuged at 4°C for 10min (3000 rpm). Plasma was subsequently stored at -80°C until analysis. Aldosterone was measured with a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California, USA). Active plasma renin concentration (APRC) was measured with a radioimmunoassay that detects the amount of angiotensin I produced per hour in the presence of excess exogenous angiotensinogen as

described previously(6) (nanograms of angiotensin I produced per liter of plasma per hour; CisBio International, France). Longitudinal quality controls were run in all assays in order to validate the results over time. The level of urinary sodium, potassium and urea were determined from the 24h-urine collections of the subjects, and assessed by the use of an automated clinical chemistry analyzer (Roche Modular Basel).

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 22.0). Data were tested for normality using histograms and the Kolmogorov-Smirnov test for normal distribution. Parametric data are presented as mean ± standard deviation (SD) in text, tables and figures, and analyzed using the Student's *t*-test or paired t-test. Nonparametric data are presented as median (25^{th}-75^{th} percentile) and analyzed using the Mann Whitney-U test or Wilcoxon Signed rank test. Gender differences in ECV and aldosterone during a low sodium diet and high sodium diet were analyzed by generalized estimating equations (GEE) analysis. Gender differences in aldosterone response were determined using GEE analysis. Statistical significance was accepted at $p<0.05$. The association between plasma aldosterone and ECV was tested using linear univariate regression analysis. For this end, plasma aldosterone was log transformed to achieve normal distribution.

RESULTS

Baseline characteristics and urinary and blood parameters

The baseline characteristics of the two groups are presented in Table 1. There were no significant differences in age, waist-to-hip ratio and BMI. Height and BSA were, as expected, significantly higher in men. Urinary albumin excretion was normal in all subjects, and did not differ between men and women (data not shown). Blood and urinary parameters during the different sodium intakes are shown in Table 2. Systolic blood pressure was higher in men during both sodium intakes (LS: 122 ± 10 vs 110 ± 9 mmHg, P=0.001; HS: 124 ± 12 vs 115 ± 8 mmHg, P=0.011). Diastolic blood pressure was higher in men during a low sodium diet (72 ± 7 vs 67 ± 7, P=0.039), but not significantly different during a high sodium diet (73 ± 8 vs 71 ± 8 mmHg, P=0.474). Urinary sodium excretion and urinary potassium excretion were equal between both groups, which reflects comparable sodium and potassium intakes during the respective dietary weeks.

RAAS hormones, extracellular volume, and their association

Aldosterone was significantly higher in men than in women during a high sodium diet intake (37 (24-63) ng/L vs 26 (10-34) ng/L, P=0.014). During a low sodium diet, this difference was no longer statistically significant (92 (72-145) ng/L vs 121 (77-154) ng/L, P=0.252; Table 2, fig 1A). APRC was significantly higher in women during both sodium intakes (LS: 9.5 (8.1-12.7) vs 5.6 (4.3-7.4) ng Ang-I/mL/h, P<0.001; HS: 4.0 (2.5-6.0) vs 2.8 (1.2-3.5) ng Ang-I/mL/h, P=0.024; Table 2). ECV data (scaled as ECV/1.73m^2 BSA) is shown in fig 1B. Men had a

significantly higher ECV than women during both sodium intakes (LS: 13.3 ± 1.8 vs 16.3 ± 2.6 L/1.73m^2, P=0.001; HS: 14.4 ± 2.2 vs 17.4 ± 2.9 L/1.73m^2, P=0.002). As expected ECV was higher during high sodium intake than during low sodium intake in both men (P=0.023) and women (P=0.006). Similar results were seen when scaling ECV to lean body mass, or to weight (data not shown). In the whole population, a higher plasma aldosterone was associated with a higher ECV during a high sodium diet (B=1.758, P=0.024, see figure 2). During a low sodium intake, this trend was borderline significant (B=1.526, P=0.103). When investigating this association per gender, no statistically significant correlations were found (data not shown). The extent of ECV reduction after sodium restriction was not correlated with the rise in aldosterone, or with blood pressure decline. Additionally, blood pressure reduction after sodium restriction was not statistically significantly correlated with the rise in aldosterone.

Adrenal response to angiotensin II infusion

To study gender differences in the regulation of aldosterone, we performed ang II infusions during a low and high sodium diet. In both men and women, the increasing doses of ang II led to a progressive increase in aldosterone levels (fig 3). In women this increase in aldosterone levels was more pronounced than in men during both sodium intakes (analysis of dose response curves by GEE analyses).

DISCUSSION

This is the first study providing a systematic comparison of aldosterone and volume status in healthy young adult men and women, under strictly standardized conditions on both a high and low sodium diet. Our data suggest that constitutive gender differences in aldosterone levels may lead to altered volume status with a higher ECV and blood pressure in men. Additionally, men have a reduced adrenal response to exogenous ang II infusion, compatible with a higher effect of endogenous ang II on adrenal aldosterone secretion(25). Therefore, the difference in aldosterone levels might be ang II mediated.

We found a higher plasma aldosterone in men than in women. This is in accordance with earlier studies, in both healthy and hypertensive subjects(9, 17). During a low sodium diet, the difference in aldosterone between men and women did not quite reach statistical significance.

The higher aldosterone in men we report could be explained by different mechanisms. First, differences in plasma potassium concentrations could influence aldosterone levels, however these were similar in men and women. Secondly, higher levels of plasma ACTH levels could stimulate additional aldosterone secretion in men, however these were not measured in the current study. Lastly, higher circulating levels of endogenous ang II, or higher adrenal sensitivity to endogenous ang II, could contribute to the higher aldosterone levels in men. However, endogenous ang II was not measured as this is notoriously difficult to interpret, and therefore we prefer assessing endogenous ang II using infusion of exogenous ang II. Indeed we found that the adrenal responses to

exogenous ang II infusion were less pronounced in men, on both sodium intakes. A lower adrenal response to exogenous ang II could be due to several factors related to greater endogenous ang II activity, such as an increased tissue concentration of endogenous angiotensin II or increased density of the angiotensin II receptor(25). Therefore, the reduced adrenal response to ang II infusion we found in men, suggests endogenous ang II facilitates the higher aldosterone levels in men. We previously reported on gender differences in ang II response – respectively of blood pressure, inversely to the current manuscript – with a larger response in men during high sodium(27). This is in line with the reciprocal response to altered endogenous ang II status between ang II sensitivity of the vascular bed and that of the adrenal gland (25).

Furthermore, we are the first to demonstrate gender differences in volume status under well-controlled conditions. We found that ECV was higher in men, both during a high and a low sodium diet. This finding was consistent when normalizing ECV to other body dimensions (i.e. length and lean body mass), marking the robustness of our data. This is in line with the results of Peters et al. who found a higher ECV (scaled to BSA) in men, in a large cohort study of healthy prospective kidney donors(20). However, in their study sodium status was not standardized, and the ECV difference did not persist when scaled to other body dimensions, or when corrected for potassium intake. Our data demonstrate an effect of sodium intake in ECV, with a rise in ECV during a high

sodium diet. This shows that it is relevant to account for sodium intake when interpreting ECV.

We found a higher systolic blood pressure in men, under well controlled conditions. Heart rate was higher in women than in men, which is in line with known literature in healthy young adults(28). In the hypertensive population, it has been well-established that blood pressure is higher in men than in pre-menopausal women(21, 23). Here, we show that in normotensive subjects this is true as well, which is in line with previous studies(12, 31). This might be mediated through gonadal hormones; testosterone levels in men might increase systolic blood pressure (SBP) (13), while estrogen levels in women might protect against high SBP(21). It has also been suggested that gender differences in sympathetic regulation of the cardiovascular system lead to differences in SBP(2). Alternatively, as we found that men have higher aldosterone levels and higher ECV, excess volume and sodium retention elicited through aldosterone might lead to higher SBP. Indeed we found an association between higher aldosterone and higher ECV. However, this association was not found in women and men separately, and was only borderline significant during a low sodium diet. While our data support the hypothesis that aldosterone causes a higher SBP in men through volume retention, intervention with an aldosterone antagonist such as spironolactone or eplerenone would provide further evidence.

We found that SBP decline after sodium restriction was subtle, and in men did not reach statistical significance. This demonstrates an intact blood pressure homeostasis in non-sodium sensitive normotensive young adults. The absence of

a visible correlation between ECV decline and SBP decline after sodium restriction further illustrates the intact feedback loop to maintain BP despite volume loss.

Our study has limitations. First, our study shows an association between gender differences in aldosterone and ECV, but cannot provide proof of the causality of this association. Second, we studied pre-menopausal women in the mid-follicular phase, caution is warranted when extrapolating our findings. Aldosterone levels and ECV are influenced by phase of the menstrual cycle, and, importantly, by menopause(3, 4). It has been shown that after menopause the gender differences in aldosterone levels and in blood pressure disappear(5, 16, 22, 32). Furthermore, we found significantly lower ARPC in men than in women, irrespective of sodium intake. This is in contrast with earlier studies, which describe a lower plasma renin in premenopausal women than in men(1, 11). This could not be explained through phase of the menstrual cycle, as renin levels were found to be lower during the follicular phase than during the luteal phase(1). As we measured APRC in two different sub-studies of the GRECO-cohort, and the measurements were performed several years apart, these results should be interpreted with caution.

In conclusion, men have a higher aldosterone, ECV and SBP than women. Furthermore, the adrenal response to ang II infusion is less pronounced in men, suggesting a higher contribution of endogenous ang II to adrenal aldosterone

295 secretion. Taken together, this well controlled physiological study gives in-depth
296 data on possible mechanisms in which gender difference in aldosterone could
297 lead to a higher ECV and blood pressure in men.

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DISCLOSURE

All authors declare no conflict of interest.

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Figure captions

Fig 1 Plasma aldosterone and extracellular volume in men and women during high and low sodium intake.

Median (75th percentile) (A) plasma aldosterone and (B) ECV during high sodium and low sodium intake in women (white boxplots), and men (grey boxplots) The whiskers represent the 10th and 90th percentile.

BSA: body surface area

** Significantly different from women (GEE analysis), p<0.05.*

 # Significantly different between low and high sodium intake (GEE analysis), p<0.05. The response of extracellular volume and aldosterone after the change in sodium intake was not significantly different between both groups (GEE analysis).

Fig 2**: **Scatterplot of distribution of extracellular volume (ECV) against plasma aldosterone during high sodium diet, and low sodium diet.

(A) High sodium diet. (B) Low sodium diet. In the whole population a higher plasma aldosterone was statistically significant associated with a higher ECV, during a high sodium intake, and borderline significant during a low sodium intake.

Fig 3: Median (with 25th-75th percentile) aldosterone concentration during angiotensin II infusion on high sodium intake and low sodium intake in women (open line) and in men (black line).

*(A) High sodium diet. (B) Low sodium diet. * significantly different from baseline*

441 *(Mann-Whitney U test), p<0.05. # significantly different from women (Mann-*

442 *Whitney U test), p<0.05. ** curves of men and women significantly different (GEE*

443 *analysis, corrected for baseline values), p<0.05.*

Table 1. Characteristics of subjects

Characteristic	Women (n = 18)	Men (n = 18)	P
Age, years	36 ± 5	31 ± 11	0.092
Waist-to-hip ratio	0.83 ± 0.04	0.85 ± 0.08	0.397
Height, cm	171 ± 5	184 ± 6	**<0.001**
BMI, kg/m^2	23.2 ± 2.7	23.2 ± 2.2	0.969
BSA, m^2	1.79 ± 0.12	2.01 ± 0.12	**<0.001**

444 *BMI, body mass index; BSA, body surface area; Data are presented as mean ±*

445 *SD. Differences between men and women are analyzed by using Student's t-test.*

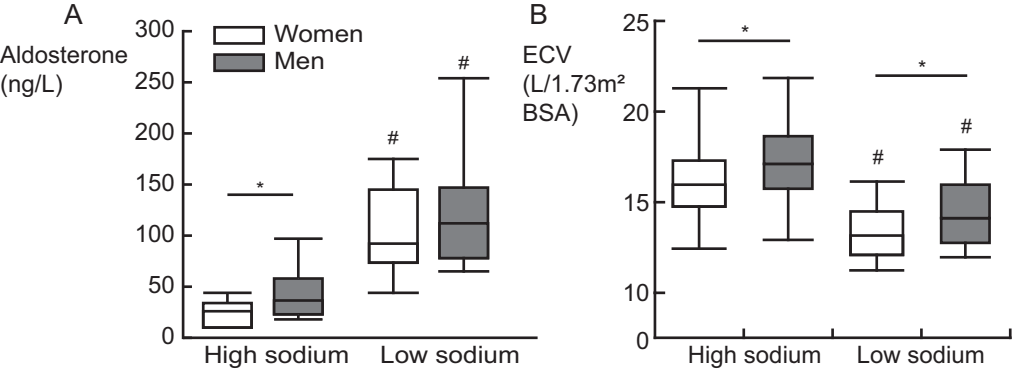
446

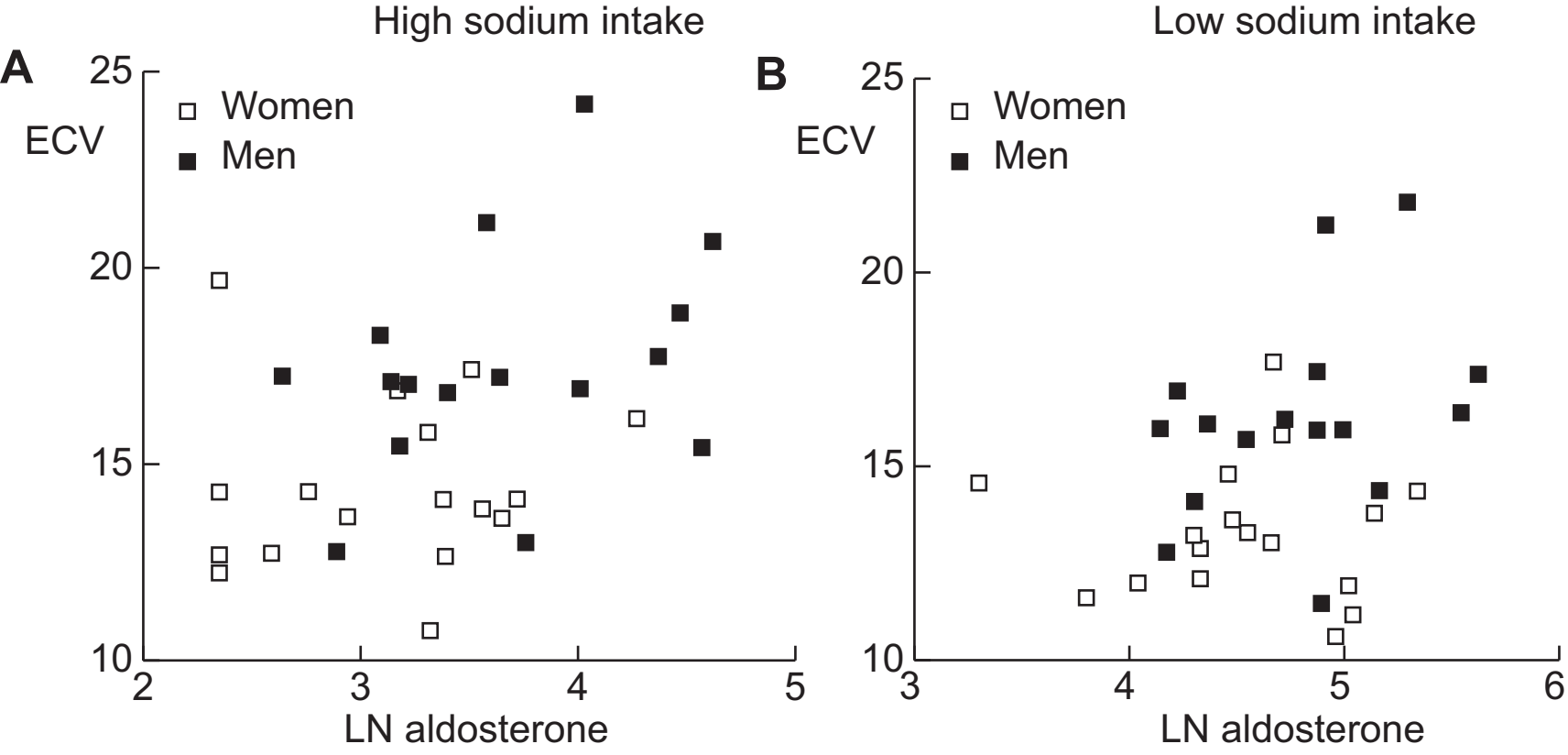
Table 2. Clinical parameters during low and high sodium intake

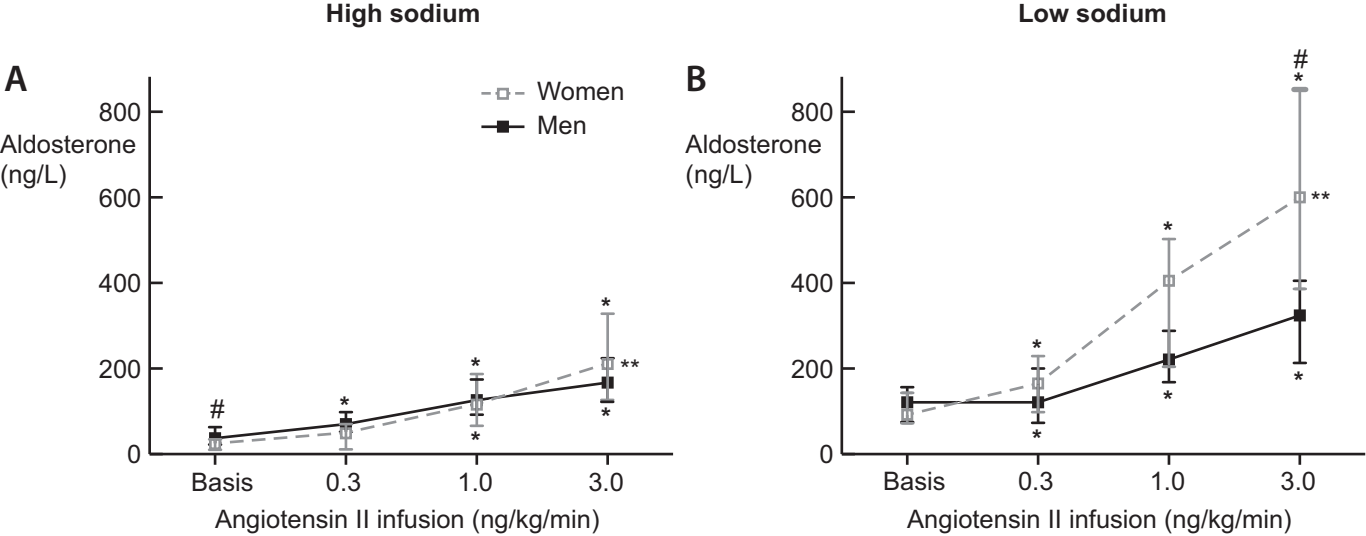
Parameter	Women (n = 18)	Men (n = 18)	P
SBP HS, mmHg	115 ± 8	124 ± 12	**0.011**
SBP LS, mmHg	110 ± 9[#]	122 ± 10	**0.001**
DBP HS, mmHg	71 ± 8	73 ± 8	0.474
DBP LS, mmHg	67 ± 7[#]	72 ± 7	**0.039**
Heart rate HS, beats/min	67 ± 8	57 ± 7	**<0.001**
Heart rate LS, beats/min	67 ± 8	60 ± 11	**0.021**
Plasma potassium HS, mmol/L	3.9 ± 0.2	3.9 ± 0.3	0.667
Plasma potassium LS, mmol/L	4.0 ± 0.2	4.0 ± 0.2	0.944
Urinary sodium HS, mmol/24h	221 ± 64	200 ± 70	0.356
Urinary sodium LS, mmol/24h	39 ± 14[#]	41 ± 27[#]	0.764
Urinary potassium HS, mmol/24h	80 ± 34	68 ± 22	0.215
Urinary potassium LS, mmol/24h	66 ± 21	76 ± 30	0.267
Urinary creatinine HS, mmol/24h	9.8 ± 1.5	15.3 ± 2.3	**<0.001**
Urinary creatinine LS, mmol/24h	9.8 ± 1.9	13.9 ± 2.9	**<0.001**
Aldosterone HS, ng/L	26 (10-34)	37 (24-63)	**0.014**
Aldosterone LS, ng/L	92 (72-145)[#]	121 (77-154)[#]	0.252
APRC HS, ng Ang-I/mL/h	4.0 (2.5-6.0)	2.8 (1.2-3.5)	**0.024**
APRC LS, ng Ang-I/mL/h	9.5 (8.1-12.7)[#]	5.6 (4.3-7.4)[#]	**<0.001**

HS, high sodium intake; LS, low sodium intake; SBP, systolic blood pressure; DBP, diastolic blood pressure; APRC, active plasma renin concentration. Data are presented as mean ± SD or median (25^{th}-75^{th} percentile). Differences between men and women are analyzed by using the Student's t-test or the Mann Whitney U test. Differences between low sodium intake vs high sodium intake are tested by using a paired t-test or Wilcoxon Signed rank test.

[#] *p<0.05 LS vs HS.*


A
Aldosterone
(ng/L)
300
250
200
150
100
50
0
Women
Men
*
#
#
High sodium
Low sodium
B
ECV
(L/1.73m²
BSA)
25
20
15
10
0
*
*
#
#
High sodium
Low sodium


High sodium intake
A
ECV
25
20
15
10
Women
Men
2
3
4
5
LN aldosterone
Low sodium intake
B
ECV
25
20
15
10
Women
Men
3
4
5
6
LN aldosterone


High sodium
A
Women
Men
Aldosterone (ng/L)
800
600
400
200
0
Basis
0.3
1.0
3.0
Angiotensin II infusion (ng/kg/min)
Low sodium
B
Aldosterone (ng/L)
800
600
400
200
0
Basis
0.3
1.0
3.0
Angiotensin II infusion (ng/kg/min)