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ORIGINAL ARTICLE

Comparison between men and women of volume regulating hormones and aquaporin-2 excretion following graded central hypovolemia

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Abstract

Central hypovolemia induced by orthostatic loading causes reno-vascular changes that can lead to orthostatic intolerance. In this study, we investigated volume regulating hormonal responses and reno-vascular changes in male and female subjects as they underwent central hypovolemia, induced by graded lower body negative pressure (LBNP). Aquaporin-2 (AQP2) excretion was measured as a biomarker for the renal system response to vasopressin. 37 young healthy subjects ($n = 19$ males; $n = 18$ females) were subjected to graded LBNP until − 40 mmHg LBNP. Under resting conditions, males had significantly higher copeptin (a stable peptide derived from vasopressin) levels compared with females. Adrenocorticotropin (ACTH), adrenomedullin (ADM), vasopressin (AVP) and brain natriuretic peptide (BNP) were not affected by our experimental protocol. Nevertheless, an analysis of ADM and BNP with the data normalized as percentages of the baseline value data showed an increase from baseline to 10 min after recovery in the males in ADM and in the females in BNP. Analysis of BNP and ADM raises the possibility of a preferential adaptive vascular response to central hypovolemia in males as shown by the normalized increase in ADM, whereas females showed a preferential renal response as shown by the normalized increase in BNP. Furthermore, our results suggest that there might be a difference between men and women in the copeptin response to alterations in orthostatic loading, simulated either using LBNP or during posture changes.

Keywords Orthostatic loading · Lower body negative pressure · Vasopressin · Copeptin · Adrenomedullin · Aquaporins

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Abbreviations

ACTH	Adrenocorticotropic hormone
ADM	Adrenomedullin
ANP	Atrial natriuretic peptide
AQP2	Aquaporin 2
AVP	Arginine vasopressin
BNP	Brain natriuretic peptide
CVP	Central venous pressure
HUT	Head up tilt
LBNP	Lower body negative pressure
RAAS	Renin–angiotensin–aldosterone system

Introduction

Hormonal responses and cardiovascular regulation

Arginine vasopressin (AVP), is a nonapeptide secreted from the posterior pituitary in response to hyperosmolality,

hypotension and hypovolemia (Bichet 2016). It controls water body homeostasis acting through the vasopressin receptors V1a, V1b and V2. AVP plays a key role in the pathophysiology of orthostatic intolerance being involved in modulating blood volume regulation (Hinghofer-Szalkay et al. 2011). In this respect, it has been shown that plasma AVP significantly increases at the point of presyncope that is characterized by the end of cardiovascular stability (Hinghofer-Szalkay et al. 2011). Repeated orthostatic intolerance episodes may occur with high incidence in older persons who often have increased plasma AVP levels although the kidney response to AVP may be decreased (Tamma et al. 2015). The actual role of AVP under conditions of orthostatic loading, which do not lead to presyncope, remains to be clarified.

Copeptin, first described in 1972 by Holwerda, can be considered a surrogate biomarker for plasma AVP (Holwerda 1972; Mohanty et al. 2015). Copepin is the C-terminal part of pre-vasopressin that is formed by a signal peptide, arginine vasopressin, neurophysin-II and copeptin. Copeptin is co-synthesized with AVP and secreted in equimolar amounts with AVP (de Bree et al. 1998). AVP consists of nine amino acids and has a molecular weight of approximately 1000 Dalton while copeptin is a 39 aminoacid glycopeptide with a molecular weight of 5000 Dalton (Nickel et al. 2012). Physiologically, copeptin may be involved in AVP maturation (Barat et al. 2004) and is more stable in vivo and ex vivo than AVP (Dobsa et al. 2013; Koch et al. 2015; Schnyder et al. 2015). In contrast, plasma AVP is unstable, largely bound to platelets and rapidly clearing making its measurement process difficult and inaccurate.

Adrenomedullin (ADM), a 52-amino acid peptide first discovered in 1993 in Phaeochromocytoma tissue, is secreted from smooth muscle cells and vascular endothelium. ADM plays a role in the regulation of blood pressure at short-term (Wong et al. 2012; Angeletti et al. 2015). Moreover, evidence also suggest that ADM is involved in the short-term adaptation to orthostatic loading at least in male subjects (Rössler et al. 1999). However, no data on the putative role of ADM in females under conditions of orthostatic loading not leading to presyncope are available.

Brain natriuretic peptide (BNP), a polypeptide secreted by the ventricles of the heart in response to excessive stretching of heart muscle cells (Hunt et al. 1995; Rademaker et al. 2005), has a physiological action similar to that of atrial natriuretic peptide (ANP). It plays important roles in decreasing systemic vascular resistance and central venous pressure as well as increasing natriuresis. It consists of 32 amino acids cleaved from a pro-hormone called N-terminal pro-hormone of BNP (NT-pro BNP). BNP plasma levels correlate with the severity of congestive heart failure. Unsurprisingly, BNP and NT-pro BNP are used as a diagnostic marker for treatment of heart failure (Suzuki et al. 2001; Kato et al. 2005; Janda et al. 2010). Despite BNP being first isolated from the brain, it is primarily secreted in the ventricles (Rose 2000). Furthermore, recent studies have shown that some patients with idiopathic orthostatic hypotension have a significant elevation of NT-pro BNP (Krishnan et al. 2015). Accordingly, BNP release is triggered by a rise in preload (Kato et al. 2005). To what extent, however, does central hypovolemia, as induced by prolonged hemorrhage or LBNP application is currently not known but needs to be investigated.

Sex, orthostatic loading and vasoactive hormones

Women have a lower orthostatic tolerance than men and a higher incidence of orthostatic hypotension as well as vasovagal syncope (White et al. 1996; Convertino et al. 1998; Waters et al. 2002; Franke et al. 2003; Fu et al. 2004; Meendering et al. 2005; Alboni et al. 2014). The exact physiological mechanisms, underling the lower orthostatic tolerance in females, are not yet clarified. AVP may play a role in orthostatic tolerance difference across sex as men show higher AVP plasma levels and a higher urine concentrating ability than women (Roussel et al. 2014). Further, the secretion pattern of AVP is dependent on the phase of the menstrual cycle (Forsling et al. 1982; Vokes et al. 1988; Stachenfeld et al. 1998). While the lower orthostatic tolerance in females may also be associated with an increased splanchnic blood pooling due to lower vasoconstriction and a lower total peripheral resistance during central hypovolemia (Jarvis et al. 2010; Russomano et al. 2015), the role of hormones across sex during central hypovolemia not leading to presyncope remains to be investigated.

Lower body negative pressure (LBNP)

To examine the effects of central hypovolemia on the human body, the use of lower body negative pressure has long been established (Goswami et al. 2008). It consists of a chamber in which the lower body of the supine subject is placed and which is then made airtight. Negative pressure is then build up within the chamber to cause a fluid shift into the lower body and thus simulate the central hypovolemia that is also induced by changes in posture from supine to standing.

In the present study, we investigated the effect of graded LBNP not leading to presyncope on plasma copeptin, AVP, ADM and BNP in both sexes. We also evaluated the effect of LBNP on Aquaporin 2 (AQP2), a water channel regulated by AVP in part excreted in the urine (u-AQP2) that can be considered a biomarker the renal response to AVP. We hypothesized that central hypovolemia—induced using graded lower body negative pressure—leads to a significant alteration in the hormones and across sex. We also hypothesized that there is a

correlation between plasma copeptin and plasma AVP and that there is also a correlation between u-AQP2 changes and changes in copeptin and AVP.

Methodology

Participants (for subject characteristics see Table 1)

Participants ($n = 19$ males; $n = 18$ females) were recruited using following specific criteria.

Inclusion criteria were ages between 18 and 35 years and height between 160 and 180 cm. Exclusion criteria were pregnancy, smokers and endurance athletes, as they are associated with reduced orthostatic tolerance (Levine et al. 1991), subjects with history of orthostatic intolerance or those with cardiovascular or renal disorders or those taking any medication affecting the cardiovascular system. The subjects were instructed to abstain from caffeine and alcohol consumption 24 h prior to the experiments. The experiments were conducted at the Medical University of Graz between 8 am and 2 pm during April and May 2015.

Experimental protocol

The experimental protocol is summarized in Fig. 1. Each experiment started with the subject being placed on the LBNP table (Fig. 2). The first stage consisted of a 30-min. Supine resting period during which the monitoring equipment was placed on the participants. LBNP was applied at the beginning with − 10 mmHg and increased by − 10 mmHg in 5-min intervals until a maximum of − 40 mmHg LBNP. This was followed by another 10-min recovery period following LBNP (Fig. 1).

Blood pressure, heart rate, stroke volume and total peripheral resistance were monitored using a Task Force® Monitor (CNSystems, Graz, Austria). Development of presyncope such as sharp drop in blood pressure or heart rate, sudden increase in sweating, nausea, and dizziness, were criteria for terminating the experiment. Furthermore, subjects could terminate the experiment using a button settled under the right hand. Subjects who experienced presyncope were excluded from hormone analysis due to the study being specifically designed to examine orthostatic loading not leading to syncope. Blood samples were taken in minutes 28, 48 and 58 from a vein in the antecubital fossa with a 17G, 1·4 · 40 mm Teflon® cannula. Urine was collected 24-h before the

Table 1 Principal characteristics of participants

	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m^2)
Males ($n = 19$)	25.85 ± 3.47	79.35 ± 9.86	181.40 ± 544	24.06 ± 2.28
Females ($n = 18$)	24.28 ± 3.43	60.94 ± 8.61	167.78 ± 5.29	21.57 ± 2.07

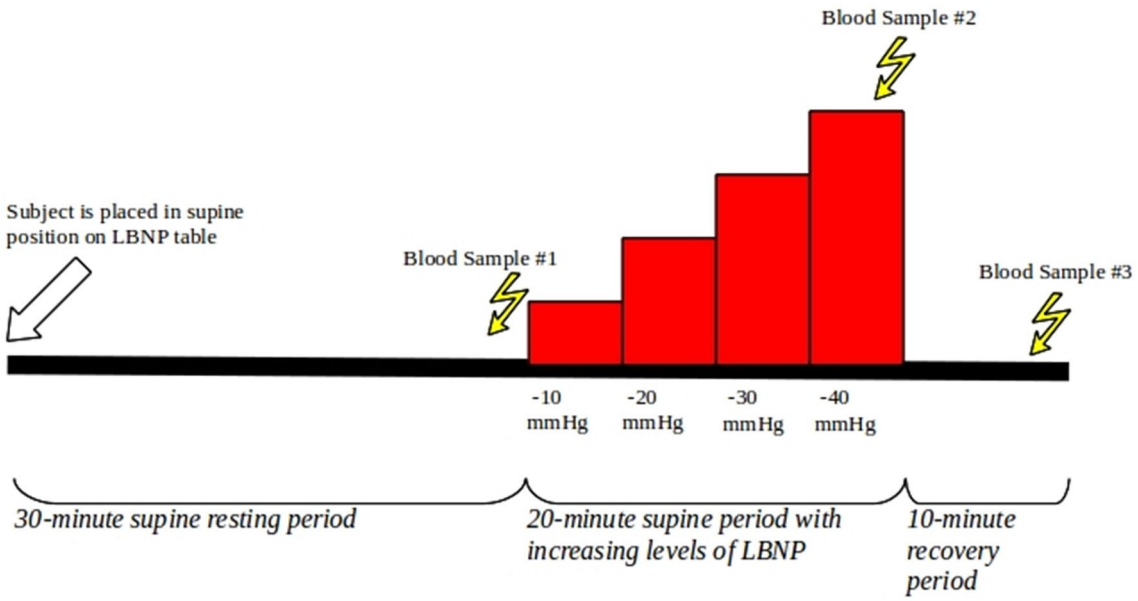

Fig. 1 Experimental protocol. The yellow arrows indicate blood collection times

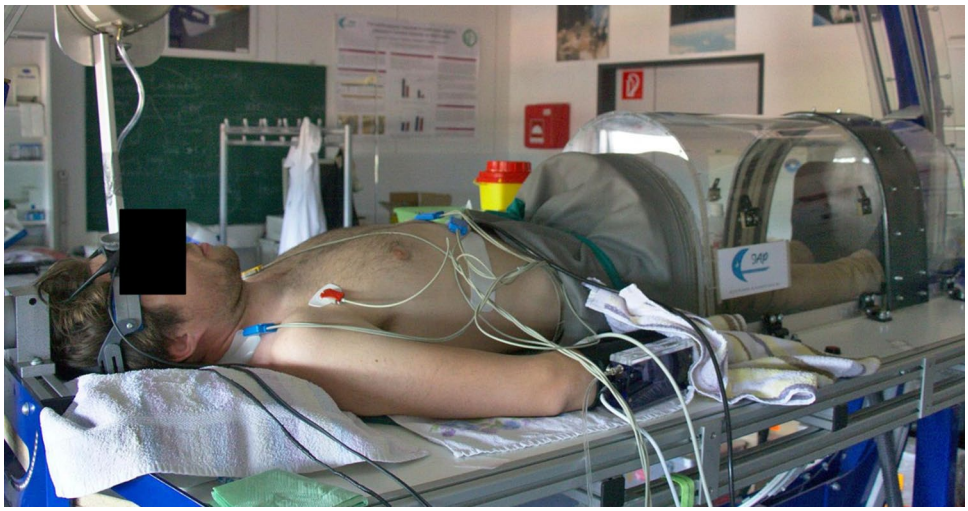

Fig. 2 Subject undergoing LBNP. Electrodes for electrocardiogram and thoracic impedance for monitoring the vital parameters are attached to the patient's chest. A device for continuous measuring of blood pressure is attached to the right arm

experiment and at the end of the recovery period following LBNP application.

Urinary AQP2 measurements by ELISA (enzyme–linked immunosorbent assay)

Urinary AQP2 excretion was measured in the urine samples by ELISA as previously described (Tamma et al. 2014). Briefly, urine samples were spun at 3000 rpm for 10 min at 4 °C to remove cellular debris in the presence of the protease inhibitors (1 mM PMSF, 2 mg/ml leupeptin, 2 mg/ml pepstatin A). 5 µl of urine sample were diluted to 50 µl in PBS containing 0.01% SDS, placed in a MaxiSorp 96-well microplate and incubated overnight at 4 °C. In parallel wells, increasing concentrations (50, 100, 200, 300, 400, 500 and 1000 pg/50 µl) of a synthetic peptide reproducing the last 15 amino acids of the C-terminal region of human AQP2 were incubated as internal standard. Wells were washed with a washing solution of PBS containing 0.1% Tween20 and incubated with blocking solution (PBS − 3% BSA) at room temperature for 1 h. 10 µg of affinity-purified anti-AQP2 antibodies were diluted in blocking solution and 50 µl of the solution was added to each well and incubated for 2 h at 37 °C. Wells were then washed with washing solution and incubated with secondary goat anti-rabbit antibodies conjugated to horseradish peroxidase for 1 h at 37 °C. After five washings with washing buffer, 50 µl of the substrate solution [2,29-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] were added to each well and incubated for 30 min in the dark at room temperature. Absorbance was measured with a microplate reader (model iMark, Bio-Rad Laboratories, Milan, Italy) at 405 nm. Urinary AQP2 excretion was expressed as fmol/mg urine creatinine. Urinary AQP2 measurements took place at the Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari Aldo Moro, Italy.

Hormone measurements

Hormones were measured from blood plasma. Blood samples were collected into chilled plastic tubes with disodium-EDTA and aprotinin. The tubes were placed on ice prior to centrifugation at 1600×*g* for 15 min at 4 °C to collect the plasma and stored at − 80 °C immediately until further processing. No sample was thawed more than two times.

ACTH and BNP were measured by a competitive inhibition enzyme-linked immunosorbent assay (Cloud-Clone Corp.) following the manufacturer's instructions with a detection limit, which was less than 4.7 pg/ml and 9.2 pg/ml, respectively. Copeptin and adrenomedullin were measured by a commercially available ELISA (Cloud-Clone Corp.) with a sensitivity less than 6.1 pg/ml and 4.9 pg/ml, respectively. AVP was measured by ELISA, the materials of which were made by ALPCO and ordered via Biomedica. Only samples measured using the same assay kit for a given hormone were included in the analysis to avoid a statistical bias due to differences among kits. All hormone measurements took place at the Physiology Division, Medical University of Graz, Austria.

Statistical analysis

For the statistical analysis, the current version 7 of the program Graphpad Prism published by Graphpad Software Inc. was used.

Hormones were analyzed using a two-way ANOVA to examine the influence of both sex and LBNP on hormone values as well as a possible interaction effect of both factors. A p value of $p = 0.05$ was deemed significant and a p value of $p = 0.01$ as highly significant. If a significant effect of sex or LBNP was observed, a D'Agostinho–Pearson normality test was performed on the data. If the normality test determined the data as non-normally distributed

($p = 0.05$), a non-parametric test was used for pairwise comparisons of the protocol stages and for inter-sex comparison. If the data passed the normality test Student's *t* test was used for pairwise comparison. Non-parametric tests were the Mann–Whitney *U* test for independent data sets, i.e., for comparison of men and women at a given protocol stage and the Wilcoxon test for paired data sets, i.e., for comparisons of the different protocol stages for a given sex. Furthermore, a regression analysis was performed for AVP and copeptin using Spearman's rank coefficient to examine the relation between these two closely connected hormones.

Results

Of the subjects examined one male and two female subjects experienced signs of presyncope and were thus excluded from hormone analysis. Hormonal data from other subjects which completed the protocol are shown in Table 2.

Table 2 Hormonal data at different timepoints during the protocol

	Baseline	End of LBNP	End of recovery
BNP			
Males ($n = 15$)	21.92 ± 16.76	19.68 ± 16.18	24.42 ± 20.21
Females ($n = 6$)	19.24 ± 10.97	24.8 ± 19.29	25.66 ± 17.45
ADM			
Males ($n = 14$)	4.49 ± 2.93	4.78 ± 2.68	5.31 ± 2.81
Females ($n = 8$)	3.15 ± 2.44	3.53 ± 2.21	3.57 ± 2.71
Copeptin			
Males ($n = 15$)	281.56 ± 116.20	236.16 ± 83.42	211.86 ± 50.13**
Females ($n = 9$)	199.70 ± 77.63	169.14 ± 68.51	152.05 ± 46.415*
AVP			
Males ($n = 14$)	2.40 ± 1.30	2.21 ± 0.69	2.16 ± 0.51
Females ($n = 7$)	1.98 ± 0.84	2.97 ± 2.11	2.68 ± 2.036
ACTH			
Males ($n = 15$)	8.832 ± 5.62	10.04 ± 4.84	10.65 ± 6.05
Females ($n = 9$)	9.085 ± 5.02	11.08 ± 7.51	8.088 ± 5.59

Difference from baseline: (*$p < 0.05$, **$p < 0.01$)

Hormones (Table 2)

AVP, ACTH, ADM and BNP

A two-way ANOVA did not yield any significant effect of protocol stage or of sex on either AVP, ACTH, ADM or BNP. No significances were seen when the data from subjects in their follicular and luteal phase were pooled together or examined separately (as two groups) and compared with each other and/or with the male subjects.

However, an analysis of ADM and BNP with the data normalized as percentages of the baseline value data showed an increase from baseline to 10 min after recovery in the males in ADM and in the females in BNP (both with $p = 0.05$) using a *t* test while no significant change was observed in the respective other sex (not shown). However, this result was obtained including the presyncopal subjects and is subject to a possible distortion due to the absence of data regarding the effect of presyncope on ADM and BNP, respectively. Furthermore, no post hoc correction was used for the *t* test.

Copeptin

Our analysis showed a significant effect of sex ($p = 0.0196$) and a highly significant effect of the protocol stage ($p = 0.0042$) on plasma copeptin (Fig. 3; Table 2). Males had a higher copeptin than females at all three protocol stages and copeptin showed a steady decrease in both sexes over course of the protocol, with arithmetic means at baseline 281.6 pg/ml in males compared to 199.7 pg/ml in females, at the end of the LBNP 236.2 pg/ml compared to 169.1 pg/ml and 211.9 pg/ml compared to 152.0 pg/ml at the end of the recovery period. These data suggest a clear downward trend of plasma copeptin levels in both sexes. The two-way ANOVA did not show a significant interaction effect of sex

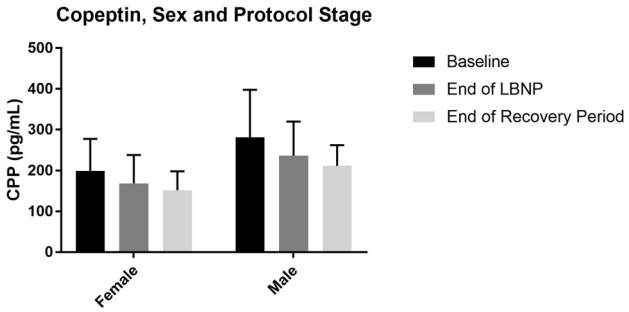

Fig. 3 Copeptin in males and females. A Wilcoxon test yielded a significant decrease between baseline and recovery for men (**$p < 0.01$) and women (*$p < 0.05$) and no significant difference between Baseline and End of LBNP or End of LBNP and Recovery. A Mann–Whitney *U* test yielded a significant sex difference at Baseline (*$p < 0.05$), End of LBNP (*$p < 0.05$) and End of Recovery (**$p < 0.01$) period. Values are expressed as means ± SD

and protocol stage on plasma copeptin. Both for men and for women, a Wilcoxon test did not yield a significant change between baseline vs end of LBNP or end of LBNP vs end of recovery. However, between baseline and end of recovery, there was a significant decrease with $p = 0.0195$ for women and a highly significant decrease with $p = 0.0034$ for men. A Mann–Whitney U test was used to compare the copeptin plasma levels of both sexes at baseline and at the end of the recovery period showing being significant with $p = 0.0270$ at baseline and highly significant with $p = 0.0035$ at the end of the recovery period, thereby indicating that the decrease in plasma copeptin over the course of the experiment was overall greater in women than in men. Thus, we observed a decrease in plasma copeptin in both sexes over the course of the experimental protocol, and a higher copeptin in men than in women, a difference which became more pronounced over the course of our experiment.

A regression analysis was also conducted to assess correlation between copeptin and AVP. Spearman's rank correlation coefficient yielded a value of 0.118 at baseline, 0.170 at the end of LBNP and 0.237 at the end of the recovery period with a p level of well over 0.1 in all three cases. These data show no significant correlation between AVP and copeptin.

Aquaporins

u-AQP2 was measured in all subjects. In basal conditions, u-AQP2 levels in the 24-h urine samples were significantly higher in females ($n = 18$, 4762 ± 338.8 fmol/mg) than in males ($n = 19$, 2756 ± 282.7 fmol/mg; $^{***}p < 0.0001$, Fig. 4a). After 10 min recovery from LBNP, u-AQP2 levels were not significantly different in both sexes, remaining significantly lower in males as compared to females after the LBNP (Fig. 4b, $^{***}p < 0.0001$ vs females initial, $^{**}p < 0.001$ vs females final, unpaired t test).

Discussion

The present study identified the renal vascular changes in response to central hypovolemia induced by graded lower body negative pressure by measuring blood volume regulating hormones in healthy males and females, at rest and after 10 min of recovery post-LBNP. No significant changes and no sex differences in ACTH or AVP were observed. Copeptin decreased from baseline to the end of the recovery period. Males had significantly higher copeptin values than females, a difference that was more pronounced at the end of the recovery period than at baseline, thereby suggesting a difference in copeptin response in our experimental protocol. BNP showed an increase from baseline to the end of the recovery period only in females and only when the data where normalized as percentages of the baseline value. Similarly, ADM showed an increase from baseline to the end of the recovery period only in males and only when the data where normalized as percentages of the baseline value. Urinary AQP2 was significantly higher in females and showed no significant change.

Under conditions of central hypovolemia, intravascular blood volume variations are sensed by vascular volume- and baroreceptors, which control the antidiuretic hormone arginine vasopressin (AVP) release, whose action induces water retention and contributes to osmotic and cardiovascular homeostasis. AVP is a peptide hormone consisting of nine amino acids, synthesized as part of a larger precursor molecule in the nuclei of the hypothalamus, stored in vesicles within the cytosol, transported down the axons and stored within the neurons close to the nerve endings in the posterior lobe of the pituitary (Laycock 2010; Koshimizu et al. 2012). During the transport, AVP is split from its precursor molecule, along with two other, larger molecules. The major stimulus for AVP release is an increase in plasma

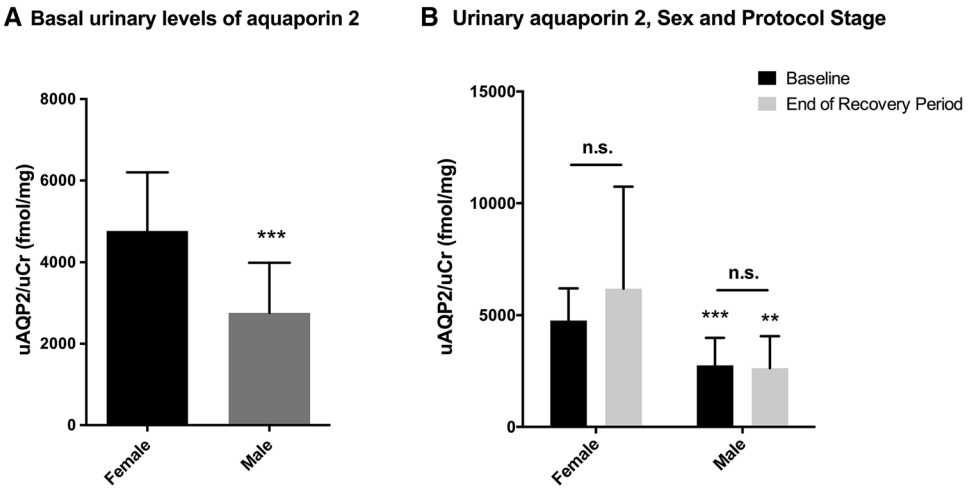

Fig. 4 Urinary levels of aquaporin 2 (uAQP2) in females and males, expressed as protein to-urinary creatinine (UrCr, in mg) ratio. **a** u-AQP2 excretion was significantly higher in females with respect to males at baseline ($^{***}p < 0.0001$). **b** u-AQP2 levels did not change significantly after 10 min recovery ($^{***}p < 0.0001$ vs females initial, $^{**}p < 0.001$ vs females final). Values are expressed as means ± SD

osmolality (1–2%) or a reduction of arterial volume (8–10%) (Wong et al. 2002). Circulating AVP is therefore orchestrated by the interplay between osmoreceptors and baroreceptors. A reduction of blood pressure detected by baroreceptor cells in the aortic arch triggers—via afferents of the glossopharyngeal and vagal nerves to the nucleus tractus solitarius and from there to the paraventricular nucleus—a release of AVP as part of the baroreceptor reflex (Norsk et al. 1993). The effects of AVP are almost exclusively mediated by three types of G-protein coupled receptors, the V1a (vascular) and V1b (pituitary) receptors which activate an inositol triphosphate pathway and the V2 (vascular and renal) receptor, which work through a cyclic adenosine monophosphate (cAMP) pathway (Koshimizu et al. 2012). In the renal collecting duct, AVP binding to the vasopressin receptor 2 (V2R) promotes water reabsorption by inducing redistribution of the water channel AQP2 from intracellular vesicles into the plasma membrane (Nedvetsky et al. 2009). This permits water entry into the cell and water exit through basolateral AQP3 and AQP4, resulting in the concentration of urine. Approximately 3% of the AQP2 expressed in renal collecting duct principal cells is excreted into urine through exosomes, small vesicles of 40–80 nm in diameter secreted into the urine by renal epithelial cells through the exocytosis of multivesicular bodies (MVBs). AQP2 excretion is proportional to its expression in the kidney and in the luminal membrane of renal collecting duct principal cells, representing a useful biomarker for water concentration diseases (Valenti et al. 2000). Specifically, an increase in AQP2 urine excretion derives from a higher translocation of the water channel to the membrane under vasopressin stimuli, whereas a reduced AQP2 excretion reflects its lower expression in the plasma membrane and the reduced renal ability to reabsorb water.

At vascular level, AVP exerts a vasoconstrictive effect, and specifically through the V1a receptors expressed in smooth muscle triggers release of Ca^{2+} ions intracellular stores resulting in contraction of muscle cells. AVP also promotes release of steroids and mineralocorticoids, stimulating ACTH release from the anterior pituitary and enhancing renin secretion as well as aldosterone action (Koshimizu et al. 2012). Under physiological conditions, AVP levels are very low (0–3 pg/ml) and even the best assays are unable to quantify them in the low range of physiological values (the lowest threshold of most immuno assays is 0.5 pg/ml) (Bankir et al. 2017). Moreover, AVP is highly unstable and rapidly degrades ex vivo even when frozen at 20 °C (Morgenthaler et al. 2006). Copeptin (CPP), which is cleaved from the C-terminal portion of the pro-vasopressin, the AVP precursor protein, has been suggested as a useful biomarker of AVP levels since it is present in an equimolar ratio to AVP and is stable even after 1 week of storage at room temperature (Repaske et al. 1997). Because of these features, using copeptin as surrogate of AVP, in this study, we found that copeptin blood levels are significantly higher in males compared with females. These data are in agreement with previous observations indicating that vasopressin plasma concentration is significantly higher in males than in females and indeed, vasopressin-mediated effects on renal and vascular targets are more pronounced in males than in females (Wang et al. 1997; Stachenfeld et al. 1998). The sex difference in copeptin levels consisted of plasma copeptin roughly 40% higher in males than in females. This almost exactly corresponds to the difference in AVP levels between the sexes reported in the literature (Crofton et al. 1986; Share et al. 1988; Roussel et al. 2014). The fact that in our experiment AVP analysis did not yield a similar result is from our point of view most likely due to its in vitro instability and the difficulties in obtaining a precise measurement.

In our study, we observed a decrease in plasma copeptin with each stage of the protocol in both the sexes. This came as a surprise to us for three major reasons. First, as outlined in the introduction copeptin is considered to be a surrogate marker for AVP. In our experiment, AVP did not show any significant change at all, while copeptin did. Second, even the sex difference known to exist in AVP plasma levels, we could not confirm this in our experiment. However, copeptin did show a significant sex difference with plasma levels being significantly higher in men at all three protocol stages. Third, LBNP causes a reduction in venous blood return to the heart, which, in turn, via a cardio renal reflex, leads to AVP release. Therefore, it was expected that copeptin would be highest at the end of LBNP with a subsequent drop at the end of the recovery period due to the venous return returning to normal supine levels after the end of LBNP. Instead we observed that in both sexes copeptin decreased steadily being highest at baseline and lowest at the end of LBNP.

Regarding the minimum LBNP level required to stimulate an AVP response current evidence is somewhat conflicting. Trimarco reported a very slight AVP increase in subjects which underwent − 10 mmHg of LBNP for 20 min, a lower stimulus than our protocol using increasing levels of LBNP starting with − 10 and ending with − 40 mmHg (Trimarco et al. 1987). Goldsmith et al. (1982) used an experimental protocol similar to ours, monitoring central venous pressure (CVP) instead of LBNP strength and reducing a baseline CVP of 7.2–3.8 mmHg for 10 min and then to 1.0 mmHg for another 10 min. Using a more recent study regarding the interaction between LBNP strength and reduction in CVP as a cross-reference, this would amount to roughly − 15 and − 30 mmHg of LBNP, respectively. Goldsmith did not observe any significant change in AVP (Goldsmith et al. 1982; Johnson et al. 2014). Furthermore, in another experiment by Roessler and colleagues (2011), even LBNP of as much as − 55 mmHg for 30-min periods with supine resting periods between them did elicit an AVP response for

the first two applications. The responses in AVP were only seen after the third LBNP period (Roessler 2011). Therefore, judging from the existing evidence it appears likely that our LBNP strength was too weak to elicit a AVP response strong enough to be detected or perhaps it could be attributed to the in vitro instability of AVP.

It has been shown that supine to erect posture change as well as ambulation increase AVP levels (Cignarelli et al. 1986; Tsuchihashi et al. 1989). Furthermore, it has been shown that plasma AVP levels adjust rapidly within approximately 10 min upon posture changes (Pump et al. 1999). We observed copeptin decreases even during central hypovolemia. We speculate that the most likely explanation for the decrease in copeptin during baseline could be due to the subjects lying in supine position for 30 min. While AVP is released in an equimolar fashion with copeptin—due to its short half-life of about 10–20 min while copeptin has a much longer half-life of about 40 min (Koch et al. 2015; Schnyder et al. 2015)—AVP should have reached a steady state even before the first blood sample was taken, while copeptin would still be decreasing. Therefore, the decrease in copeptin may be attributed to the assumption of supine posture at the beginning of the experiment. Furthermore, the significant decreases in copeptin measured after 10 min recovery from LBNP might be due to the expected fluid shifting back to the upper body. It should be noted in this respect that mean copeptin as well as mean AVP at the end of LBNP was already lower than at baseline thus suggesting that LBNP levels used in this study did not affect copeptin levels. This is further supported by the fact that we did not observe a significant correlation between AVP and copeptin, something which would be expected immediately after a peak in equimolar AVP and copeptin release.

An interesting observation is the fact that in our experiment the decrease in copeptin from baseline to end of recovery was more significant in men than in women; a more significant decrease from baseline to the end of recovery in men ($p < 0.01$) than in women ($p < 0.05$) was seen as was a more pronounced sex difference at the end of recovery than at baseline ($p < 0.05$ vs $p < 0.01$). As the two-way ANOVA did not show a significant interaction effect of sex and protocol stage, it is difficult to ascertain whether plasma copeptin responses differ across sex or whether the observed differences were caused by LBNP application or due to 30 min supine posture assumption at the beginning of the experiment. Generally speaking, our data confirm that copeptin is of value as a surrogate marker for AVP measurement but that differences in plasma half-life must be considered with a sufficient baseline period necessary to allow plasma copeptin to reach a steady state. This might be is of special importance in the design of future experimental protocols.

Analysis of u-AQP2, an index of the renal response to AVP, revealed that while under basal condition u-AQP2 levels in the 24-h urine samples were significantly higher in females than in males. However, after 10 min recovery from LBNP, u-AQP2 levels were not significantly different in both sexes; these levels remained significantly lower in males with respect to females after the LBNP. Although a decrease in u-AQP2 in parallel to AVP decrease was expected, it is possible that 10-min recovery time was not sufficient to detect significant differences with respect to the resting conditions. Alternatively, it is possible that under LBNP condition, there may be a dissociation between AQP2 excretion and AVP as has been reported in particular pathological conditions (Ranchin et al. 2010). However, as this study was carried out in healthy subject, no detectable u-APQ2 levels could have arisen due to the shorter time to recovery.

The cardiac natriuretic peptides, ANP and BNP are important regulators of the homeostatic control of blood pressure, salt and water balance. They can be considered biomarkers of cardiac health due to the close relationship between plasma concentrations of these peptides and "cardiac load". In our experiment, BNP did not show any significant alteration when the raw data were analyzed. Only when the data were normalized as percentage of the baseline values, and presyncopal subjects were included a significant increase at the end of the recovery period was seen but only in the female subjects.

ADM, a vasodilator peptide hormone, largely expressed throughout the cardiovascular system plays a key role in the regulation of cardiovascular function (Nishikimi et al. 2013). ADM has a powerful vasodilatory effect especially in the kidney, brain and lung and several vasoconstrictive agents have been shown to elevate its concentrations, among them norepinephrine, aldosterone and angiotensin II. These observations suggest that ADM may serve to counterbalance the vasoconstrictive effects of these substances (Nagaya et al. 2000; Wong et al. 2012). An interesting finding in this respect is the observation that ADM increases cerebral baroreceptor reflex response in rats (Ho et al. 2008). Furthermore, its other effects include increasing the cardiac output, apparently via an inotropic effect (Nagaya et al. 2000).

It has been reported that head-up-tilt (HUT) over 30° leads to a significant increase of plasma ADM within as little as 2 min correlating with the degree of HUT, thus indicating that ADM might play a significant role in quick adaptation to orthostatic loadings (Roessler et al. 1999). In Roessler's study, ADM continued to increase until the end of HUT after 30 min and rapidly returned to baseline levels within 3 min after the return to supine position. The physiological stimulus by which the ADM release was triggered in this study could be central hypovolemia caused by upright posture or LBNP detected via tension receptors in the right atrium and triggering ADM release in a cardio renal mechanism highly like the one triggering the release of AVP. It

must be said, however, that ADM's effects on cardiovascular regulation are complex and still poorly understood. In our experiment, no significant alterations of ADM were shown, even though LBNP reduces venous return to the heart and therefore should at least in theory influence ADM release. It should be noted that if presyncopal subject was included there was a significant increase, but only in the males. At the same time, including the presyncopal subjects and normalizing the BNP data led to a significant increase only in the females. This raises the question whether males and females might respond to alterations in orthostatic loading via different mechanisms. Unfortunately, the fact that these observations do not appear when presyncopal subjects were excluded makes it necessary to conduct further research to sufficiently test this hypothesis.

Conclusions and future directions

Overall, the analysis of volume regulating hormones suggests that central hypovolemia induced by LBNP should stimulate afferent inputs to the brain leading to AVP release while soon after the end of central hypovolemia an inhibition of AVP release should take place. The fact that this was not observed in our study is consistent with most evidence indicating this response taking place only under conditions of higher levels of central hypovolemia than the ones caused by the LBNP strength we used. Analysis of BNP and ADM raises the possibility of a preferential adaptive vascular response to central hypovolemia in males as shown by the normalized increase in ADM, whereas females showed a preferential renal response as shown by the normalized increase in BNP. Furthermore, our results suggest that there might be a difference between men and women in the copeptin response to alterations in orthostatic loading, simulated either using LBNP or during posture changes. Future studies should examine the role of ADM and BNP in both sexes under conditions of orthostatic stress and the level of central hypovolemia needed to elicit an AVP response.

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Author contributions NG designed the experiments wrote the manuscript. JR designed the experiments, performed the experiments, and analyzed the data. ADM performed the experiments and analyzed the data. BB performed the experiments and analyzed the data. AR performed the experiments and analyzed the data. MC performed the experiments and analyzed the data. MR performed the experiments and analyzed the data. AR performed the experiments and analyzed the data. NGDS critical reading of the manuscript. GT performed the experiments and analyzed the data. FCS designed the experiments and wrote the manuscript. GV designed the experiments and wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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