Effects of tilting and volume loading on plasma levels and urinary excretion of relaxin, NT-pro-ANP, and NT-pro-BNP in male volunteers

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Submitted 10 November 2003; accepted in final form 25 February 2004

Heringlake, Matthias, Carsten Heide, Ludger Bahlmann, Wolfgang Eichler, Horst Pagel, Peter Schmucker, Ragnhild Wergeland, Franz Paul Armbruster, and Stephan Klaus. Effects of tilting and volume loading on plasma levels and urinary excretion of relaxin, NT-pro-ANP, and NT-pro-BNP in male volunteers. J Appl Physiol 97: 173–179, 2004. First published February 27, 2004; 10.1152/japplphysiol.01196.2003.—The polypeptide relaxin (RLX) has been suggested to play a role in cardiorenal integration and to be related to the natriuretic peptide system. We hence examined the effects of variations in thoracic blood volume and intravenous volume loading on plasma and urinary RLX levels and associated changes in natriuretic peptide levels in healthy men. Two groups of eight subjects were randomly tilted into a 15° feet-down or a 15° head-down position. Ten volunteers were cross-over subjected to an infusion of 15 ml/kg of 0.9% NaCl (over 60 min) or control during an observation period of 10 h. Blood and urine were sampled at timed intervals. RLX, Nt- and prohormones of atrial natriuretic peptide (NT-pro-ANP), and Nt-H- and terminal prohormones of brain natriuretic peptide (NT-pro-BNP) were determined by enzyme, radio-, and electrochemoluminescence immunoassays, respectively. NT-pro-ANP levels (in percentage of baseline levels) were higher (P < 0.05) during the head-down (124 ± 13%) than during the feet-down position (82 ± 6%). NT-pro-BNP and RLX were not affected by tilting. Volume loading induced a short-lasting increase in plasma NT-pro-ANP, a delayed increase in plasma NT-pro-BNP, had no effect on plasma RLX, and induced a parallel increase in urine flow, renal excretion of sodium, RLX, and NT-pro-BNP. It is concluded that variations in thoracic blood volume in healthy men are not associated with variations in plasma RLX. Increased urinary RLX and NT-pro-BNP excretion during volume loading suggest renal production and a possible role of kidney-derived RLX and brain natriuretic peptide in sodium homeostasis in men.

THE POLYPEPTIDE RELAXIN (RLX), formerly known as a pregnancy hormone (6), has recently gained interest as a neurohumoral mediator in human heart failure (5). Dschietzig and coworkers (5) have shown that the plasma levels of RLX are increased in relation to the severity of myocardial disease, that the heart is a relevant source of RLX in cardiac failure patients, and that plasma RLX concentrations in these patients are related to left ventricular filling pressures. Toth and coworkers (22) have shown that RLX increased the secretion of atrial natriuretic peptide (ANP) in an isolated rat heart model. RLX receptors have been observed in rat atria (18); additionally, this peptide has been shown to increase glomerular filtration rate and renal plasma flow in conscious rats (3).

These findings are suggestive that RLX may play a role in cardiorenal integration and that the plasma levels of this hormone may be related to changes in cardiac filling pressures and are possibly linked to the natriuretic peptide system. No data are available that assess whether the plasma levels of RLX in healthy individuals are influenced by postural changes and acute fluid loading as factors that are known to modify natriuretic peptide release (10, 20).

Hence we hypothesized that changes in thoracic blood volume (TBV), induced either by tilting or by intravenous volume loading, might induce concomitant changes in plasma and urinary RLX levels in healthy male volunteers. The activity of the natriuretic peptide system in blood and urine was determined by analyzing the course of the Nt-H-terminal prohormones of ANP (NT-pro-ANP) (21) and brain natriuretic peptide (BNP; NT-pro-BNP) (16). Additionally, urinary excretion of urodilatin (Uro; UUro), a kidney-derived member of the natriuretic peptide family system, was studied (8).

MATERIALS AND METHODS

The investigation conforms to the principles outlined in the Declaration of Helsinki. Following approval by the institutional review board and informed, written consent, 16 healthy male, nonsmoking volunteers (age: 27 ± 4 yr; weight: 82 ± 11 kg; height: 184 ± 6 cm) on a customary sodium diet were studied. All subjects participated in the tilting protocol, and 10 of the volunteers were additionally enrolled in the volume-loading protocol. The studies were performed in a temperature-controlled laboratory after an overnight fast. After arrival at the laboratory at 0800, subjects were placed in the supine position and equipped with a 16-gauge venous cannula, which allowed blood sampling without congestion. All subjects received a standard breakfast at 0915 (2 slices of toast, marmalade, 3 ml/kg water).

Tilting Protocol

Sixteen volunteers were randomly divided into two groups of n = 8 and studied in different body positions for 2 h each. After a resting period in the supine position, subjects were either tilted to a 15° head-down position or to the feet-down position, were brought back into the supine position, and afterward were tilted into the opposite direction.

Hemodynamics [heart rate (HR), electrocardiogram, mean arterial blood pressure, automated oscillometric sphygmomanometer] were...
samples were immediately spun for 10 min at 3,400 rpm at 4 °C. Blood and urine were collected every 15 min and averaged for respective time periods.

**Blood and Urine Analyses**

Blood and urine analyses were performed on standardized hospital analyzers. Blood for hormone analysis was sampled in EDTA tubes containing 5,000 units aprotinin (Trasylot, Bayer, Germany) and lithium-heparin tubes (for clinical chemistry), as appropriate. Blood and urine samples were immediately spun for 10 min at 3,400 rpm at 4°C. Supematants were stored at −80°C until analysis. Plasma and urine electrolytes (flame photometrically) and creatinine (enzymologically) were determined on standard hospital analyzers.

Renal functional parameters [fractional excretion of sodium (FE Na) and creatinine (enzymologically)] were calculated according to standard formulas. Urinary excretion of hormones was expressed as the ratio between the hormone and the creatinine concentration.

**Determination of RLX**

RLX was determined by a sandwich-type enzyme immunoassay, as described previously (1, 5). In short, this assay is based on two specific polyclonal antibody raises in rabbits against recombinant human gene H2-RLX. The detection limit of this assay is 1.2 pg/ml. The standard range is 1.2–250 pg/ml. Recovery in plasma is 97 ± 4.5%. The intra-assay coefficient of variance at 15.3 pg/ml was 10.2% (n = 12). The assay has a cross-reactivity of <0.01% with insulin, insulin-like growth factor, LH, and FSH.

**Determination of NT-pro-ANP**

NT-pro-ANP was determined by a competitive-binding radioimmunoassay with magnetic solid-phase technique, in a modification of Sundsfjord et al. (21), by using the same rabbit anti-rat pro-ANP polyclonal serum, human pro-ANP-(1–30) from Peninsula Laboratories (Bachem, St. Helene, UK) as the standard, and iodined pro-ANP-(1–30) purified by HPLC for radiolabeling. To achieve high sensitivity and good precision, Dynabeads M280 with sheep antirabbit IgG (Dynal Biotech, Oslo, Norway) as solid-phase and second antibody were used. The coefficients of variance at 425, 1,163, and 2,490 pmol/l were 7.5, 3.7, and 3.4%, respectively. The detection limit was 30 pmol/l.

**Determination of NT-pro-BNP**

NT-pro-BNP was determined by an electrochemoluminescence immunoassay (Elecys pro-BNP sandwich immunoassay; Roche Diagnostics, Basel, Switzerland) on Elecsys 2010 (16). The mean intra-assay variance was 4.3% (range: 2.7–5.9% for plasma samples with a concentration between 7.6 and 2,732 pmol/l, with an interassay variance of 3.2%). The lower detection limit was 0.6 pmol/l.

**Determination of Uro**

Uro was determined by a RIA for human Uro (Immundiagnostik, Bensheim, Germany), as described previously in detail (15). The antiserum does not cross-react with ANP-(99–126), NT-pro-BNP, or BNP. The sensitivity of the method was 10.5 pg/ml, and the intra- and interassay variabilities were 8.1 and 9.7%, respectively.

**Statistical Analyses**

Data in the text are given as means ± SD, data in Figs. 1–4 are given as means ± SE. Significance was set to P < 0.05.

**Tilting protocol.** With respect to high variance at baseline, hormone and LAD data were normalized by calculating relative changes compared with the baseline levels that were set to 100%. Thus normalized, the data were analyzed parametrically. Between-group differences were determined by ANOVA for repeated measures. Bonferroni correction was not used. Intraindividual differences compared with normalized baseline values were analyzed with paired Student’s t-test.

**Volume-loading protocol.** Due to the small sample size, calculations on the raw data were performed nonparametrically. Intraindividual differences between the control and the volume group were analyzed by Friedman’s test followed by Wilcoxon’s matched-pairs test. Bonferroni correction was not used. Intraindividual differences during the observation period were determined by Wilcoxon’s matched-pairs test. Spearman’s rank correlation test was used for correlation analyses.

Table 1. *Hemodynamic data in 16 healthy volunteers undergoing tilting maneuvers*
RESULTS

Tilting Protocol

Hemodynamics. Neither HR nor arterial blood pressure showed significant intra- or interindividual changes throughout the observation period (Table 1). A significant difference in LAD was observed during the second tilting period (Table 1). Clinical chemistry. Plasma sodium, potassium, and creatinine at baseline were not different between tilting groups (plasma sodium: group 1, 138 ± 2 mmol/l; group 2, 139 ± 2 mmol/l; plasma potassium: group 1, 3.8 ± 0.3 mmol/l; group 2, 3.6 ± 0.2 mmol/l; plasma creatinine: group 1, 75 ± 9 μmol/l; group 2, 81 ± 8 μmol/l). No significant between-group variations in these parameters were observed throughout the observation period (data not shown).

Plasma hormone levels. The course of normalized NT-pro-ANP, NT-pro-BNP, and RLX levels is depicted in Fig. 1. NT-pro-ANP levels were higher during HD than during FD in the second tilting period. NT-pro-BNP levels increased over time until 1500 but were not different between both groups. No significant interindividual variations were observed in plasma RLX levels. The intraindividual course of this hormone was comparable in both groups, showing a surge in normalized RLX levels at 1500 compared with baseline and an increase from 1500 to 1600 back to baseline levels.

Volume-loading Protocol

Hemodynamics. No significant within- and between-group differences were observed in HR and mean arterial blood pressure (Table 2). A small, albeit significant, increase in LAD was observed after the saline infusion from 1100 to 1200. Thereafter, LAD decreased back to baseline levels (Table 2). Clinical chemistry. Plasma sodium, potassium, creatinine, and hematocrit at baseline were not different between the control and the volume-loading protocol (plasma sodium: control, 139 ± 2 mmol/l; volume loading, 140 ± 2 mmol/l; plasma potassium: control, 3.6 ± 0.3 mmol/l; volume loading, 3.4 ± 0.7 mmol/l; plasma creatinine: control, 80 ± 11 μmol/l; volume loading, 76 ± 9 μmol/l; hematocrit: control, 40.7 ± 2.0%; volume loading, 40.1 ± 1.8%).

Plasma hormone levels. The plasma levels of NT-pro-ANP, NT-pro-BNP, and RLX are depicted in Fig. 2. In the volume-loading group, NT-pro-ANP levels increased immediately with a peak 2 h after the infusion. A moderate increase was also observed in the control group. However, after 1200, NT-pro-ANP levels in this group were not different from baseline levels. NT-pro-BNP showed a delayed increase up to the end of the observation period in both groups. NT-pro-BNP levels in the volume-loading group were signiﬁcantly higher than in the control group after 1300. No significant variations or between-group variations in RLX levels were observed.

Renal function parameters. Urine flow (UF) and FE\textsubscript{Na} showed a moderate increase from 0800 to 1000. After sodium infusion, a further and more pronounced increase was observed (Fig. 3). Thereafter, UF decreased back to preinfusion levels, whereas FE\textsubscript{Na} remained elevated until the end of the observation period. Creatinine clearance did not change throughout the observation period (Fig. 3).

Urinary excretion of hormones. Urinary excretion of NT-pro-BNP (U\textsubscript{NT-pro-BNP}), RLX (U\textsubscript{RLX}), and U\textsubscript{Uro} is given in Fig. 4. Determination of NT-pro-ANP was only possible in a minority of urine samples; hence, no calculations on this parameter were performed. U\textsubscript{NT-pro-BNP} and U\textsubscript{RLX} increased significantly from 0800 to 1000 and further, to reach a peak at 1200. Thereafter, urinary hormone excretion decreased but remained elevated above baseline levels. U\textsubscript{Uro} showed a comparable course like U\textsubscript{NT-pro-BNP} and U\textsubscript{RLX}; however, due to a high variance and the fact that U\textsubscript{Uro} concentrations in two
RELAXIN, ANP, AND BNP DURING TILTING AND VOLUME LOADING

DISCUSSION

The present study was designed to determine whether variations in TBV and intravenous volume loading with normal saline may induce changes in the plasma levels and U_Uro and accompanying variations in the NT-pro-ANP and NT-pro-BNP and the renal excretion of Uro in healthy volunteers. The findings of this study clearly show that, in contrast to NT-pro-ANP and NT-pro-BNP, plasma levels of RLX are altered neither by acute changes in body position nor during acute volume loading. Interestingly, U_RLX was stimulated by volume infusion and correlated with UF and sodium excretion, suggesting renal production of this peptide and a possible role in fluid and sodium homeostasis.

An increasing number of studies point to a role of RLX in cardiac failure (5, 7). Dschietzig and coworkers (5) have shown an increase in plasma levels and myocardial mRNA expression of RLX in relation to the severity of disease in patients with cardiac failure. They showed that the heart is a major source of circulating RLX during this condition and that the plasma concentrations of this peptide are related to cardiac filling pressures (5), suggesting that the plasma levels of RLX are related to the pathophysiological events of congestive heart failure (CHF): myocardial distension and fluid overload. We thus hypothesized that the secretion of RLX may also be increased by myocardial stretch and may hence, comparable to ANP (10, 20), also react to variations in posture or acute volume loading in healthy individuals.

However, based on the findings of the present study, we have to reject this hypothesis. During the tilting protocol, plasma RLX levels were not affected by posture changes. Hormone levels were significantly lower at 1500 compared with baseline values and increased rapidly back to baseline levels from 1500 to 1600 in both groups. With respect to the highly comparable course in both groups, this may not be explained by circadian rhythmicity and hence warrants further study. Additionally, no changes in plasma RLX levels were detectable during volume loading, despite the long observation period. Taken together, these findings more or less rule out that acute variations in TBV are associated with changes in plasma RLX levels in healthy, male subjects.

The plasma RLX levels observed in our study were tremendously higher (30–50 pg/ml) than the normal values (2.5 pg/ml) and the values for severe CHF patients (17 pg/ml) in the study mentioned above (5). Of note, analyses of RLX levels in our study were determined with the same immunoassay and in the same laboratory and were highly reproducible in comparison between the control and the volume-loading group. Because none of the subjects in our study had overt heart failure, according to physical examination, and low NT-pro-BNP levels also ruled out even discrete myocardial disease, on a first glance, these findings questions the data presented by Dschietzig et al. (5). However, in contrast to these investigators, we used aprotinine for inhibition of peptide degradation during blood sampling, a fact that may explain the higher plasma RLX levels in our study.

The course of NT-pro-ANP during tilting and the accompanying changes in LAD show that the changes in position were sufficient to induce relevant changes in these parameters and hence in TBV, at least during the second tilting period. However, no tilting-induced changes in other hemodynamic parameters or in NT-pro-BNP were detectable.

It is long known that dietary sodium loading increases the plasma levels of ANP (11) and BNP (13). This may account for the discrete and short-lasting increase in NT-pro-ANP levels in the control group during the fluid-loading protocol. Comparably, the moderate increase in NT-pro-BNP levels during the tilting protocol in both groups and the increase in NT-pro-BNP levels throughout the fluid-loading protocol in the control group are supposed to be an effect of the standardized breakfast given at 915.

Table 2. Hemodynamic data in 10 healthy volunteers in the control and the volume-loading group

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Volume loading</th>
</tr>
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<tbody>
<tr>
<td>HR, Beats/min</td>
<td></td>
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</tr>
<tr>
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<td>67±8</td>
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<tr>
<td>1000</td>
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<tr>
<td>MAP, mmHg</td>
<td></td>
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</tr>
<tr>
<td>0900</td>
<td>83±6</td>
<td>83±5</td>
</tr>
<tr>
<td>1000</td>
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</tr>
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LAD, mm

<table>
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<th>Volume loading</th>
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<tbody>
<tr>
<td>0900</td>
<td>3.7±0.4</td>
<td>3.7±0.4*</td>
</tr>
<tr>
<td>1000</td>
<td>3.9±0.4</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td>1100</td>
<td>3.7±0.4</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>1200</td>
<td>3.6±0.3</td>
<td>3.6±0.4</td>
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</table>

Values are means ± SD (absolute data). *Intraindividual difference in comparison with baseline levels: P < 0.05.
levels would have been missed. Consequently, the delayed increase in NT-pro-BNP in our study is an important new finding. Besides the general implications for our understanding of the natriuretic peptide system, this may be relevant for the interpretation of NT-pro-BNP levels as a hormonal marker for the severity of myocardial failure at the bedside, because sodium-containing fluids are frequently used in the clinical setting.

Fig. 2. Volume-loading protocol. The course of plasma levels of NT-pro-ANP (A), NT-pro-BNP (B), and RLX (C) in 10 healthy volunteers during control (○) and fluid loading (□). Data are given as means ± SE. *Between-group difference, \( P < 0.05 \) (Friedman’s test followed by Wilcoxon’s matched-pairs test). †Significant \( (P < 0.05) \) difference compared with baseline values (Wilcoxon’s matched-pairs test).

Fig. 3. Volume-loading protocol. The course of urine flow (UF), fractional excretion of sodium (FE_{Na}), and creatinine clearance (C_{Cr}) in 10 healthy volunteers in the fluid-loading group. Data are given as means ± SE. †Significant \( (P < 0.05) \) difference compared with baseline values. ‡Significant \( (P < 0.05) \) difference compared with values at 1000 (before sodium infusion) (Wilcoxon’s matched-pairs test).
As expected, volume loading induced an immediate rise in UF and FE\textsubscript{Na} without accompanying changes in glomerular filtration rate. Interestingly, these effects were accompanied by significant increases in the U\textsubscript{RLX} and U\textsubscript{NT-pro-BNP}, whereas only a marginal increase in U\textsubscript{Uro} was observed. Of note, the hormonal excretion of hormones was adjusted for UF by expressing the data relative to creatinine excretion. Hence the excretion pattern cannot be explained simply as a washout in face of an increase in tubular flow and UF. Moreover, the different course of plasma NT-pro-BNP and the unchanged plasma RLX levels suggest that the peptides detected in urine were kidney derived.

RLX as well as BNP have previously been reported to be detectable in urine (19, 23); however, in contrast to Uro, which has been shown to be produced only in the kidney and which has been suggested to play a major role in human sodium homeostasis (8), information on the physiological role of urinary RLX and BNP is sparse. Urinary RLX has been shown to be modified by estrogen therapy in women and has thus been used to quantify the induction of urinary vasoactive markers during contraceptive use (19). Urinary BNP has been shown to be increased in patients with renal (23) and heart failure (17). No data on a possible coupling between renal function and U\textsubscript{RLX} and urinary excretion of BNP are available.

RLX has been shown to increase glomerular filtration rate and sodium excretion in conscious rats, even in the nonpregnant state (2–4). However, in contrast to the well-described distribution of natriuretic peptide receptors in the kidney (14), the distribution of RLX receptors in the kidney is unknown. RLX has been convincingly shown to induce renal vasodilatation and hence a decrease in renal vascular resistance (2, 4). However, it is not known whether additional factors, i.e., tubular effects leading to an inhibition of sodium reabsorption, or other humoral systems are involved.

The different time course of plasma and urinary NT-pro-BNP levels during volume loading more or less excludes that the NT-pro-BNP levels detectable in urine are of plasmatic origin. Comparably, the lack of correlation between U\textsubscript{NT-pro-BNP} and UF and the minor relationship between U\textsubscript{NT-pro-BNP} and FE\textsubscript{Na} contradict that the immediate renal response to volume loading may be attributable to variations in plasma NT-pro-BNP levels. Consequently, it is more plausible that the increase in sodium excretion is either related to changes in central hemodynamics (LAD) or ANP and/or mediated by the urinary derivatives NT-pro-BNP or RLX.

In conclusion, the data of the present study show that acute variations in body position and acute intravenous volume

### Table 3. Correlation analyses between urine flow and fractional excretion of sodium vs. plasma level and urinary excretion of NT-pro-ANP, NT-pro-BNP, relaxin, and urodilatin in healthy volunteers throughout the observation period in the fluid-loading group

<table>
<thead>
<tr>
<th></th>
<th>UF</th>
<th>FE\textsubscript{Na}</th>
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<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT-pro-ANP</td>
<td>0.34*</td>
<td>0.42*</td>
</tr>
<tr>
<td>NT-pro-BNP</td>
<td>NS</td>
<td>0.27*</td>
</tr>
<tr>
<td>Uro</td>
<td>NS</td>
<td>0.5*</td>
</tr>
<tr>
<td>Uro</td>
<td>0.47*</td>
<td>NS</td>
</tr>
</tbody>
</table>

Spearman’s rank correlation test calculated on the pooled data from 10 healthy volunteers during an observation period of 10 h after an infusion of 15 ml/kg isotonic saline. UF, urine flow; FE\textsubscript{Na}, fractional excretion of sodium; NT-pro-ANP, NH\textsubscript{2}-terminal prohormones of atrial natriuretic peptide; NT-pro-BNP, NH\textsubscript{2}-terminal prohormones of brain natriuretic peptide; RLX, relaxin; Uro, urodilatin. Correlations are given as “Spearman’s rho.” *Significant (P < 0.05) difference compared with baseline values. †Significant (P < 0.05) difference compared with values at 1000 (before sodium infusion) (Wilcoxon’s matched-pairs test).
loading do not alter plasma levels of RLX in healthy male volunteers. This contrasts with pronounced effects of these maneuvers on the plasma levels of the NT-pro-ANP and NT-pro-BNP and suggests that, despite being increased in relation to filling pressures in CHF, RLX does not react like an acute filling-pressure-dependent hormone in healthy humans. However, the association between U_{RLX} and urinary excretion of sodium suggests a role of kidney-derived RLX in human sodium and fluid homeostasis that warrants further study.

ACKNOWLEDGMENTS

We thank Josef Schenk from Roche Diagnostics, Penzberg, Germany, for providing the NT-pro-BNP kits. We deeply acknowledge the help of Charlotte Neubert from Immunodiagnostik AG, Bensheim, and our institutional statistician, Dr. Michael Hipp. We also thank Frank Hoffmann from Philips Ultrasound, Germany for providing the echocardiography device.

REFERENCES


