Do alterations of endogenous angiotensin II levels regulate erythropoietin production in humans?

S. Freudenthaler,¹ P. Benöhr,² A. Grenz,² T. Selzer,¹ T. Schmidt,¹ K. Mörike,¹ H. Osswald² & C. H. Gleiter^{1,3}

¹Abteilung Klinische Pharmakologie, ²Abteilung Experimentelle Pharmakologie Institut für Pharmakologie und Toxikologie, Universitätsklinikum Tübingen, and ³Koordinierungszentrum Klinische Studien am Universitätsklinikum Tübingen, Tübingen, Germany

Aims Recent evidence suggests a potential role of angiotensin II in the physiological regulation of erythropoietin (Epo) production. While the administration of exogenous angiotensin II (AII) has been used so far to study its effects, the role of endogenous AII has remained unclear.

Methods To alter endogenous AII in humans experimentally we used furosemide bolus injection as a short-term (study 1) and dietary salt as a long-term modulator (study 2). In an open crossover design, 12 healthy male volunteers received furosemide (F) 0.5 mg kg⁻¹ intravenously or placebo (P) in random order (study 1). With the same design, 12 volunteers received high-salt (HS), normal-salt (NS) and low-salt (LS) diet (study 2). Plasma renin activity (PRA) was analysed along with AII. Inulin and paraaminohippurate (PAH) clearances were used to indicate glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively.

Results While F stimulated AII and PRA and decreased GFR and RPF significantly, no concomitant alteration of Epo was observed [AUCEpo: placebo 5709 ± 243 (% of baseline × h), furosemide: 5833 ± 255 (% of baseline × h); 95% confidence interval (CI) -608.4, 856.0; P = 0.73]. F decreased GFR (from 103.6 ± 4.0 to 90.6 ± 4.8 ml min⁻¹ 1.73 m⁻²; 95% CI 1.1, 24.9; P < 0.05), but not RPF (study 1). Correspondingly, LS stimulated and HS decreased AII and PRA significantly. HS increased GFR and RPF. Again, Epo concentrations were not affected (AUCEpo: normal sodium 44 ± 6.7 mIU × day ml⁻¹, low sodium 39 ± 2.4 mIU × day ml⁻¹, high sodium 48.5 ± 6.1 mIU × day ml⁻¹; normal salt/low salt 95% CI -11.9, 21.9, P = 0.54; normal salt/high salt 95% CI -14.4, 23.3, P = 0.63; study 2).

Conclusions We conclude that, at least in the physiological setting in healthy volunteers, increased concentrations of endogenous AII may not be a major factor of Epo regulation.

Keywords: angiotensin II, erythropoietin, furosemide, healthy volunteers, high salt-diet, low salt-diet

Introduction

A number of findings support the hypothesis that angiotensin II (AII) may be one mediator in the control of erythropoietin (Epo) production. Fisher *et al.* showed that infusing anaesthetized dogs with angiotensin AII leads to an increase in the plasma levels of Epo [1]. However, in this paper Epo concentrations were not measured directly but with the polycythemic mouse assay. A similar effect on Epo production was also seen after indirectly increasing AII levels with the help of a Goldblatt clamp of the

renal artery [1]. Gould *et al.* demonstrated that, after rats are exposed to hypoxia, there is a positive correlation between renin activity, renin substrate, angiotensin I and Epo levels [2]. In rats with normal renal function ACE inhibitors may induce anaemia or reduce polycythemia with an accompanying decrease of erythropoietin [3].

Hints of a role for angiotensin in the control of Epo secretion stem from observations in human studies. In systematic examinations we have shown that theophylline increases Epo concentrations in humans after a controlled stimulus with haemorrhage [4–6]. These findings may be interpreted as an indirect effect, via the increase of plasma renin activity (PRA) with a subsequent increase of AII levels as it occurs during theophylline infusion. Based on these findings, in a later investigation we administered fenoterol alone and in combination with the (specific and

Correspondence: Dr C. H. Gleiter, Abteilung Klinische Pharmakologie, Universitätsklinikum Tübingen, Otfried Müller-Str. 45, D-72076 Tübingen, Germany. E-mail: christoph.gleiter@med.uni-tuebingen.de

Received 31 January 2003, accepted 30 April 2003.

selective) AT1-receptor blocker losartan to human subjects [7]. It was shown that fenoterol induces an increase of PRA and Epo concentrations. The latter was completely inhibited by administration of the AT1-receptor blocker losartan, indicating an involvement of AII in the regulation of Epo production.

A further hint on the role of AII as a mediator of Epo production stems from the observation that continuous treatment with angiotensin-converting enzyme (ACE) inhibitors is able to lower Epo concentrations in healthy volunteers with intact renal function [8] and patients with post-transplant erythrocytosis (PTE) [9, 10]. Other clinical experiments demonstrated that ACE inhibition therapy reduced altitude polycythemia which is considered an Epo-dependent form of polycythemia [11]. Losartan has been demonstrated to reduce haematocrit and haemoglobin concentrations in patients with chronic obstructive pulmonary disease and secondary erythrocytosis [12]. Also anaemia in hyporeninaemic hypoaldosteronism has been linked to reduced Epo levels [13].

ACE inhibitors have also been demonstrated to reduce renal Epo release acutely from the affected kidney in patients with renal artery stenosis [14]. Others could not demonstrate an acute effect by a single dose of enalapril on serum Epo in patients with end-stage renal disease [15, 16].

Further systematic investigations of ours have shown that exogenous AII dose-dependently increases peak Epo concentrations in humans by >300% of baseline. The application of the AT1-receptor blocker losartan inhibited the rise of Epo levels, whereas ACE inhibitors did not [17, 18]. Later, these findings were confirmed by others [19]. Interestingly, Naomi *et al.* [20] have shown in essential hypertensive patients with salt loading that Epo concentrations correlate positively with haematocrit. They were able to demonstrate slightly higher Epo levels, which, however, were still in the normal range.

As it stands there are many but equivocal hints that point towards a role of angiotensin in the production of Epo and AII may be involved in its physiological regulation. This has never been investigated systematically. We therefore tested the hypothesis that physiological modulation of endogenous AII concentrations alters Epo levels in humans. We chose furosemide bolus injection as a short-term modulator stimulation (study 1) and high salt intake for 7 days as a long-term modulator inhibition of plasma renin activity, respectively (study 2) of Epo.

Methods

Subjects

Study 1 Twelve healthy nonsmoking male volunteers (age 22–34 years, body mass range 62–95 kg). The study

was conducted according to an open, randomized, placebo-controlled, cross-over design. After inclusion the subjects were randomly allocated to the following treatments: furosemide (Lasix®, 0.5 mg kg⁻¹ body weight; i.v. bolus injection over 5 min) or placebo (Sterofundin®, 0.9% NaCl solution, 5 ml i.v. bolus injection over 5 min).

Study 2 Twelve healthy nonsmoking male volunteers (age 22–35 years, body mass range 60–97 kg). The study was conducted according to an open, randomized, controlled, cross-over design. After inclusion the subjects were randomly allocated to the following diet periods of 7 days each separated by a washout period of at least 7 days: normal sodium (NS, mean NaCl intake 10.38 g day⁻¹), high sodium (HS, mean NaCl intake 22.37 g day⁻¹), low sodium (LS, 0.78 g day⁻¹); normal sodium (NS, mean NaCl intake 10.38 g day⁻¹), high sodium (HS, mean NaCl intake 22.37 g day⁻¹), low sodium (LS, mean NaCl intake 0.78 g day⁻¹).

All study subjects gave their written informed consent to participate in one of the trials. Their health status was checked on the basis of physical examination, blood chemistry (including iron and ferritin), urine analysis, and ECG tracings. No drugs were allowed for 2 weeks before or during the trials. The volunteers were not allowed to stay at an altitude of ≥1000 m above sea level (including air travel) 2 weeks before and during the trials. Blood, plasma or thrombocyte donors, during the previous 3 months were excluded. No xanthine derivative-containing food or beverages were allowed from 1 day before and during the study. The study protocols were approved by the Ethics Committee of the Medical Faculty of the University of Tübingen.

Protocols

Study 1 Each volunteer was randomly allocated to 2 study days receiving furosemide and placebo, respectively. On each study day kidney function [glomerular filtration rate (GFR); renal plasma flow (RPF)] was measured by inulin- and para-aminohippurate (PAH) clearance under steady-state conditions. Urine and blood samples were taken before and after bolus injection of furosemide and placebo, respectively (= 0 h). Water intake of the volunteers was restricted to 75% of the excreted volume. Blood samples were taken at -3, 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 and 48 h after injection of the study medication for determination of Epo, AII, PRA, electrolytes and haematocrit and haemoglobin, inulin and PAH. Samples for PRA and AII were prechilled and kept on ice during their handling, centrifugation was done at 4 °C. Bestatin (Bühlmann Laboratories, Schönenbuch, Switzerland), an angiotensinase inhibitor,

was added to the samples for the measurement of AII concentrations.

The subjects remained in supine position from the moment they entered the ward until the end of the clearance measurements (-3 h to $+3$ h) and were deprived of all exogenous stimuli. RR and HF were measured continuously every hour from the bolus infusion up to 4 h, then parallel to the blood sampling. There was a washout period of at least 1 week between the study days.

The volunteers had to ingest 2500 ml day^{-1} of fluid for 4 days before the study day. Balanced body fluid and electrolyte content was checked 2 days before the study day by 24-h urine collection. The subjects had to produce a urine volume of at least 2000 ml with sodium and potassium concentration excretions within the normal range of our laboratory (sodium $80\text{--}240\text{ mmol } 24\text{ h}^{-1}$; potassium $25\text{--}80\text{ mmol } 24\text{ h}^{-1}$).

Basal GFR was calculated as a mean from the three baseline measurements. Urine from the first collection period after medication was discarded and GFR calculated from the subsequent three periods (see clearance methods below).

Study 2 The volunteers received during 7-day periods a normal, high and low sodium diet in random order. There were washout periods of at least 7 days between the diet periods. The entire diet was prepared by the central catering services of the University Hospital Tübingen. High sodium diet was achieved with an additional sodium load (100 mg kg^{-1} body weight day^{-1}) using gelatine capsules filled with the appropriate amount of sodium chloride (prepared by the pharmacy of the University Hospital Tübingen). Compliance was verified with urine sodium measurements from 24-h urine collected on day 6 of the respective period of diet. Blood sampling for PRA and AII was done on days 1 and 7 at 08.30 h after a 30-min supine rest. Blood samples for Epo measurements were taken on days 1–7 at 08.30 h. Examination of clearances was done as reported below. Samples of the respective parameters were handled identically as in study 1. Blood pressure (RR) and heart rate (HR) were measured during blood sampling on the respective study days.

Inulin and PAH clearance

For clearance investigations, two 20-G peripheral venous cannulas were inserted into volunteers in the antecubital vein on either side for infusion of marker substances and contralateral blood sampling. Following collection of blood and urine samples as blanks and for determination of safety laboratory parameters, a priming dose of 18 ml m^{-2} body surface area (BSA) inulin (Inutest®; Fre-

senius Pharma Austria, Linz, Austria) and 4 ml m^{-2} BSA PAH (p-aminohippuric acid sodium salt®; Clinalfa AG, Merck, Laeufelfingen, Switzerland) was administered in 250 ml 0.9% saline at a rate of 500 ml h^{-1} over 30 min. Thereafter, PAH ($1.6\text{ ml h}^{-1}\text{ m}^{-2}$ BSA) and inulin ($5\text{ ml h}^{-1}\text{ m}^{-2}$ BSA) in 250 ml 0.9% saline were continuously infused at a rate of 50 ml h^{-1} . After 60 min of equilibration, three 30-min baseline clearance periods were performed, during which fluid losses were substituted orally by medicinal water [containing (mg l^{-1}) Na^+ 7.8, K^+ 3.1, Ca^{2+} 104.2, Cl^- 12.0, HCO_3^- 402.6]. Subsequently, an oral water load of 1500 ml was ingested within 30 min and another four clearance periods were performed.

Urine samples were collected at the end of each clearance period by spontaneous voiding. Blood samples were drawn at mid period. During clearance periods, subjects remained in a supine position except for voiding.

Analytical methods

Blood chemistry, including iron and ferritin, haemoglobin and haematocrit, as well as creatinine and sodium and potassium in urine and plasma were measured in the Department of Clinical Chemistry, University Hospital, Tübingen.

As in earlier studies [4–7, 18], the following commercially available assay kits were used. Epo in plasma was analysed using a monoclonal enzyme-linked immunosorbent assay (ELISA, catalogue no. 500; Medac, Hamburg, Germany), PRA was measured with a radioimmunoassay kit for angiotensin I ^{125}I -RIA (RENK P2510; Sorin, Saluggia, Italy). AII in plasma was determined using a radioimmunoassay (number 40-6005; Nichols Institute Diagnostics, San Juan Capistrano CA, USA).

Concentrations of inulin and PAH in plasma and urine were determined by a colorimetric assay. Clearances were calculated according to standard formulae (see below) and normalized for a body surface area of 1.73 m^2 for GFR and RPF.

Plasma and urinary electrolyte concentrations were determined by flame photometry. Mean daily sodium intake was calculated estimated from the average sodium excretion of two consecutive 24-h urine collections, with 58.4 mmol of urinary sodium corresponding to 1 g of ingested sodium chloride.

Calculations

Study 1 The area under the Epo serum concentration-time curve [AUC Epo ($0\text{--}48\text{ h}$)], using Epo concentration data adjusted to the percentage of baseline and the area under the PRA [AUCPRA ($0\text{--}3\text{ h}$)] and AII [AUC AII ($0\text{--}3\text{ h}$)] serum concentration time-curve were

calculated from the original data using the trapezoidal rule. $C_{\max}$ Epo was taken from the curve of erythropoietin serum concentration *vs.* time. Clearance (CL) was calculated as $CL = (U \cdot V_{\text{urine}}) / P \times t$, with U being the concentration of the respective compound in urine, V_{urine} the volume of urine per 24 h, P the mean plasma concentration of the respective compound from measurements at the midpoint of each urine collection period, and t the urine sampling interval.

Study 2 The area under the Epo serum concentration–time curve [AUC_{Epo} (0–7 days)], using Epo concentration data, was calculated using the original data from the trapezoidal rule.

Statistical analysis

For either study the sample size calculation was performed assuming that the smallest expected difference of AUC_{Epo} is 50%. This was regarded a clinically relevant change of Epo concentrations based on the results of our earlier studies [17, 18]. The standard error was assumed to be 33%. Accordingly, a $P < 0.05$ with a power of 0.9 was expected with 12 volunteers using a cross-over design.

Values are expressed as mean $\pm$ SEM. Ninety-five percent confidence intervals (95% CI) were calculated for differences of sample means where meaningful.

Study 1 Data were compared by paired t -tests. $P < 0.05$ was considered statistically significant.

Study 2 Values of angiotensin II concentrations and PRA were compared intra-individually by paired t -test (day 1 *vs.* day 7). Comparisons between groups were analysed by one-way ANOVA (analysis of variance) followed by post hoc t -tests with α -adjustment according to Dunnett. $P < 0.05$ was considered statistically significant.

Results

Study 1

Time courses of haematocrit and haemoglobin (Table 1), RR and HR were always in the normal range and were not altered to a clinically relevant extent throughout the

entire study (other data not shown). In Table 2, values of GFR and RPF before and after injection of the study medication are given. After injection of placebo, GFR and RPF remained unchanged, injection of furosemide provoked a statistically significant decrease of GFR (before furosemide $103.6 \pm 4.0 \text{ ml min}^{-1} 1.73 \text{ m}^2$; after furosemide $90.6 \pm 4.8 \text{ ml min}^{-1} 1.73 \text{ m}^2$; difference of means $13.0 \text{ ml min}^{-1} 1.73 \text{ m}^2$; 95% CI 1.1, 24.9; $P < 0.05$) whereas no significant changes of RPF were detected.

In Figure 1 the time course of AII concentrations for placebo and furosemide treatment is given. Baseline concentrations were similar (placebo $4.03 \pm 0.66 \text{ pg ml}^{-1}$; $7.58 \pm 2.69 \text{ pg ml}^{-1}$). After placebo injection, AII concentrations remained unchanged, whereas after furosemide injection $C_{\max}$ increased about 3.4-fold and was statistically significantly higher compared with placebo (placebo $4.59 \pm 0.69 \text{ pg ml}^{-1}$; furosemide $15.57 \pm 2.30 \text{ pg ml}^{-1}$; difference of means 10.9 pg ml^{-1} ; 95% CI 6.58, 15.21; $P < 0.01$).

The time course of PRA values for F and P is depicted in Figure 2. Baseline values were similar after both treatments (placebo $0.60 \pm 0.07 \text{ ng ml}^{-1} \text{ h}^{-1}$; furosemide

Table 1 Haematocrit and haemoglobin.

		<i>Placebo</i>	<i>Furosemide</i>
Haematocrit study 1			
Before (vol. %)		39.5 ± 0.8	39.7 ± 0.6
10 h after start of treatment (vol. %)		37.8 ± 0.9	42.1 ± 0.6*
Haemoglobin study 1			
Before (mg dl ⁻¹)		13.9 ± 0.3	14.0 ± 0.2
10 h after start of treatment (mg dl ⁻¹)		13.5 ± 0.3	14.6 ± 0.2
	<i>Normal sodium</i>	<i>High sodium</i>	<i>Low sodium</i>
Haematocrit study 2			
Day 1 (vol. %)	41.6 ± 1.2	40.6 ± 0.7	43.3 ± 0.6
Day 7 (vol. %)	39.3 ± 0.8	38.9 ± 0.6	41.2 ± 0.7*
Haemoglobin study 2			
Day 1 (mg dl ⁻¹)	14.4 ± 0.4	14.0 ± 0.3	15.0 ± 0.2
Day 7 (mg dl ⁻¹)	13.7 ± 0.3	13.6 ± 0.2	14.5 ± 0.2

Study 1, Before and after 10 h of treatment (at the maximum of erythropoietin concentrations); study 2, day 1 and day 7 of treatment; mean $\pm$ SEM; * $P < 0.05$.

Table 2 Glomerular filtration rate (GFR) and renal plasma flow (RPF) before and after injection of the study medication placebo or furosemide.

	Placebo		Furosemide	
	Before injection	After injection	Before injection	After injection
GFR ($\text{ml min}^{-1} 1.73 \text{ m}^2$)	97.6 ± 3.9	98.6 ± 2.09	103.6 ± 4.0	$90.6 \pm 4.8^*$
RPF ($\text{ml min}^{-1} 1.73 \text{ m}^2$)	545.1 ± 33.1	542.7 ± 24.9	537.9 ± 33.5	606.4 ± 35.8

Mean $\pm$ SEM; * $P < 0.05$ before *vs.* after injection.

Figure 1 Time course of angiotensin II (AII) concentrations after bolus injection of furosemide (●) and placebo (▼) (mean $\pm$ SEM). A significant four-fold increase of $C_{\max}$ AII (furosemide *vs.* placebo) could be detected (* $P < 0.01$).

Figure 2 Time course of plasma renin activity (PRA) after bolus injection of furosemide (●) and placebo (▼) (mean $\pm$ SEM). As expected, a significant five-fold increase of PRA (furosemide *vs.* placebo) could be detected (* $P < 0.01$).

$0.65 \pm 0.10 \text{ ng ml}^{-1} \text{ h}^{-1}$). The PRA time course remained unchanged after injection of placebo. After furosemide a five-fold increase of PRA occurred with a statistically significant higher $C_{\max}$ compared with placebo (placebo $0.60 \pm 0.07 \text{ ng ml}^{-1} \text{ h}^{-1}$; furosemide $2.93 \pm 0.88 \text{ ng ml}^{-1} \text{ h}^{-1}$; difference of means $2.32 \text{ ng ml}^{-1} \text{ h}^{-1}$; 95% CI 0.42, 4.22; $P < 0.05$).

The time course of Epo concentrations is given in Figure 3. Baseline Epo levels were similar before either treatment (placebo $9.62 \pm 1.32 \text{ mIU ml}^{-1}$; furosemide $9.15 \pm 0.73 \text{ mIU ml}^{-1}$). No differences of Epo concentration–time course and $C_{\max}$ Epo between furosemide and placebo treatment could be detected.

No statistically significant differences of AUC_{Epo} between the respective treatments were observed [placebo 5709 ± 243 (% of baseline $\times$ h), furosemide 5833 ± 255 (% of baseline $\times$ h); difference of means 123.8 (% of baseline $\times$ h), 95% CI -608.4 , 856.0; $P = 0.73$).

Study 2

Time courses of haematocrit and haemoglobin (Table 1), RR and HR were always in the normal range and were not altered to a clinically relevant extent throughout the entire study (other data not shown). Sodium excretion, GFR and RPF on day 7 of the respective diets is given

Figure 3 Time course of erythropoietin (Epo) concentrations after bolus injection of furosemide (●) and placebo (▼), respectively (mean $\pm$ SEM). No significant differences were detected between the two treatments.

in Table 3. As expected, during low sodium treatment sodium excretion was significantly lower and during high sodium excretion significantly higher compared with normal sodium intake (low sodium *vs.* normal sodium: difference of means $164.1 \text{ mmol } 24 \text{ h}^{-1}$, 95% CI 118.4, 209.8; $P < 0.05$; high sodium *vs.* normal sodium: differ-

Table 3 Sodium excretion, glomerular filtration rate (GFR) and renal plasma flow (RPF) at the end of the respective diet phases.

	Normal sodium	High sodium	Low sodium
Sodium excretion (mmol 24 h ⁻¹)	177.4 ± 19.8	382.4 ± 41.7*	13.3 ± 2.9*
GFR (ml min ⁻¹ 1.73 m ⁻²)	84.1 ± 3.0	95.8 ± 3.6*	77.2 ± 3.2
RPF (ml min ⁻¹ 1.73 m ⁻²)	423.9 ± 15.2	488.0 ± 19.9*	447.3 ± 15.1

Mean ± SEM; **P* < 0.05 vs. normal sodium.

Figure 4 Plasma renin activity (PRA) values before the start (day 1) and on day 7 of the respective diet (mean ± SEM; **P* < 0.05 day 1 vs. day 7). High sodium (●); normal sodium (▼); and low sodium (■).

Figure 5 Angiotensin II concentrations before the start (day 1) and on day 7 of the respective diet (mean ± SEM; **P* < 0.05 day 1 vs. day 7). High sodium (●); normal sodium (▼); and low sodium (■).

ence of means 205.0 mmol 24 h⁻¹, 95% CI 109.3, 300.7; *P* < 0.05). GFR and RPF were significantly higher during high sodium treatment compared with normal sodium (GFR: difference of means 11.6 ml min⁻¹ 1.73 m², 95% CI 1.9, 21.3; 95% CI; *P* < 0.05; RPF: difference of means 64.1 ml min⁻¹ 1.73 m²; 95% CI 12.3, 116.0; *P* < 0.05), whereas no differences between low sodium intake and normal sodium could be detected.

A comparison of PRA values before start (day 1) and at the end of the respective diet (day 7) is depicted in Figure 4. Baseline values were similar before all diets. During normal sodium diet no significant changes were detected (day 1 vs. day 7). A slight but statistically significant decrease of PRA was detected during high sodium intake (day 1 vs. day 7; difference of means 0.51 ng ml⁻¹ h⁻¹; 95% CI 0.11, 0.91; *P* < 0.05), whereas low sodium intake led to a statistically significant 4.1-fold increase of PRA (day 1 vs. day 7; difference of means 2.60 ng ml⁻¹ h⁻¹; 95% CI 1.25, 3.96; 95% CI; *P* < 0.01).

In Figure 5 a comparison of AII concentrations between diet day 1 (immediately before start of diet) and day 7 (end of diet) is given. Baseline concentrations were similar in all diets. No significant differences were

detected during normal sodium intake, while during high sodium diet a statistically significant decrease of AII concentrations could be shown (day 1 vs. day 7; difference of means 2.85 pg ml⁻¹; 95% CI 0.89, 4.81; *P* < 0.01). A statistically significant 3.1-fold increase of AII concentrations was detected during low sodium diet (day 1 vs. day 7; difference of means 9.84 pg ml⁻¹; 95% CI 6.91, 12.78; *P* < 0.001).

Baseline Epo levels were similar in all groups (Figure 6). No changes of Epo concentrations occurred during any diet phase (Figure 6). No statistically significant differences of AUC_{Epo} between the respective diets could be detected (normal sodium 44 ± 6.7 mIU × day ml⁻¹, low sodium 39 ± 2.4 mIU × day ml⁻¹, high sodium 48.5 ± 6.1 mIU × day ml⁻¹; difference of means normal salt/low salt 5.01 mIU × day ml⁻¹, 95% CI -11.9, 21.9, *P* = 0.54; difference of means normal salt/high salt 4.5 mIU × day ml⁻¹, 95% CI -14.4, 23.3, *P* = 0.63).

Discussion

A number of clinical observations have produced circumstantial but equivocal evidence that the renin-angiotensin

Figure 6 Time course of erythropoietin (Epo) concentration during the respective diets. No significant differences were detected between the respective diets. No changes occurred during the diets. High sodium (●); normal sodium (▼); and low sodium (■).

system (RAS) may be involved in the regulation of Epo production in humans in various diseases [9, 11, 13]. Inhibitors of ACE [9] or AT1-receptor antagonists [12, 21] such as losartan have been shown to decrease haemoglobin, haematocrit and Epo in patients with post-transplant erythrocytosis. This topic is still a target subject of many preclinical and clinical investigations [e.g. 22].

From our earlier controlled experiments in humans [4, 5, 7, 17, 18] we concluded that AII can be a direct stimulant of Epo production in healthy volunteers. In systematic investigations we [17, 18], and subsequently others [19], found that AII has a dose-dependent effect on Epo production which can be blocked by AT1-receptor antagonists but does not change insulin-like growth factor levels [18]. Infusion rates of AII, which did not alter mean arterial pressure (MAP), were able to increase Epo concentrations by up to a maximum of approximately 230% and elevated AII concentrations three-fold compared with baseline values [18]. The order of magnitude of the increase of Epo concentrations in humans under i.v. AII administration is very similar to that of hypoxia (exposure to 13% O₂ or altitude levels of about 4000 m) [5, 23, 24].

It remains, however, unclear whether these observations of an effect of exogenous AII on Epo production can be explained either by direct mediation via AT1-receptors on Epo-producing cells or by other signal pathways altered by increased AII concentrations, e.g. vasoconstriction and a subsequent local intrarenal hypoxia signal resulting in an increase in Epo-production by the kidney. Recently published data from anaesthetized rats show an influence of angiotensin II on total renal, cortical and medullary blood flow measured by laser-

Doppler-flux in anaesthetized rats without an effect on systemic blood pressure [25]. Such data cannot be obtained *in vivo* from humans.

This problem relates to effects of AII on renal work (i.e. sodium reabsorption) by changes of intrarenal blood flow. Furosemide is known to inhibit sodium reabsorption, thus possibly reducing renal work. Eckardt *et al.* [26] have shown in normoxic rats that treatment with furosemide (20 mg kg⁻¹ body weight) increases mean Epo concentrations by approximately 40% 3 h after furosemide application. It should be noted that they used a five-fold higher dose than we were able to administer in humans in this trial. Thus, such an effect may be dose dependent, but this has never been systematically investigated. Also, the same authors demonstrated under carbon monoxide inhalation (0.1%) that furosemide, hydrochlorothiazide and amiloride did not alter the hypoxia-induced Epo response. However, acetazolamide (500 mg kg⁻¹) reduced the Epo response under hypoxia. This was interpreted by them as a hint that renal work of the proximal tubule may be linked to the regulation of Epo production. Acetazolamide did, however, not affect Epo production under normoxia. The meaning of their animal data can hardly be interpreted with respect to the physiology of humans. Our own work in rats showed that increased breakdown of ATP as it occurs under tubular workload does not affect Epo production [27]. Our investigations in humans showed that AII increased Epo concentrations accompanied by a reduction of creatinine clearance and fractional sodium excretion – a situation where less tubular work can be assumed [17].

The present studies were carried out to examine whether changes of endogenous AII levels within the physiologically occurring range may influence Epo secretion and alter renal haemodynamics.

In study 1 we examined the effect of a loop diuretic on plasma levels of AII and subsequently on Epo production. It has been shown that diuretics may produce short-lasting increases in AII concentrations by a similar order of magnitude in healthy volunteers [28, 29] and patients [30], as we saw in our experiments with i.v. AII [17, 18]. Indeed, in the present study we achieved a similar order of magnitude of acute AII increases by furosemide application as in our earlier experiments [18] with a suppressor dose of AII. However, in contrast to these experiments, in the present study Epo production remained unchanged.

Basal concentrations of Epo in the present study 1 were similar in all treatment groups and were in accordance with the values obtained in our previous investigations [4, 5, 7, 17, 18]. They were in the normal range for healthy subjects according to the Epo ELISA used (2.5–25 mU ml⁻¹) [31]. The course of PRA, GFR and

RPF values after furosemide application was in agreement with findings in the literature [32]. PRA baseline values were in the expected range for the supine position ($0.2\text{--}2.7\text{ ng ml}^{-1}\text{ h}^{-1}$) in both groups [33]. Basal AII plasma concentrations were in the expected range ($18\text{--}113\text{ pg ml}^{-1}$) for the assay used after 3 h in the supine position at 0 h and remained unaltered in the placebo group. The time course behaviour of PRA and AII concentration in both groups was as expected from the pharmacology of the system. In contrast to our earlier findings with exogenously infused AII, the furosemide-induced bolus which led to a short-term increase of AII concentration did not alter Epo concentrations. This would mean that a short but several-fold increase of in II concentration by several hundred percent after a furosemide bolus administration does not play a major role in the physiological regulation of Epo production.

In study 2 basal values for Epo, PRA and AII were also in the normal range, as in study 1. Sodium homeostasis reached a plateau within 7 days after a constant salt intake. As expected, GFR and RPF increased under high sodium diet and PRA and AII were slightly reduced. GFR, RPF, PRA, and AII remained unaltered during normal sodium intake, whereas sodium excretion decreased and PRA and AII increased under low salt diet. There was no influence on Epo production by sodium load. It appears unlikely that GFR changes affect Epo clearance to a significant extent, as only 10% of Epo is excreted via the kidney [34]. The experiment in humans does not allow detailed analysis of different intrarenal variations of blood flow and possible intra-organ responses of Epo secretion. Schmid *et al.* examined the effect of variation of dietary salt intake on AII type 1 receptors in rat organs [35]. They were able to show that high and normal salt intake did not influence the expression of AT1-receptor subtypes. However, low salt (0.2 mg g^{-1} body weight) diet led in the kidney to a downregulation of the abundance of AT1A receptor mRNA but not that of AT1B mRNA, with a nadir between days 5 and 10 of this diet. Thus, low salt diet appears to negatively influence AT1A receptor gene expression selectively. It should be noted that this investigation did not separate single anatomical structures of the kidney, but measured the overall effect of salt on the number of AT subreceptor types in kidney tissue. As we have shown that the effect of AII infusion can be blocked with the AT1A selective blocker losartan [17, 18], the findings of Schmid *et al.* may be relevant to the interpretation of our experiments in humans [35]. It may be possible that our low salt diet over 7 days led to a downregulation of the AT1A receptor subtype in the subjects' kidneys, thus blunting or even blocking the response to endogenous AII by Epo, despite the sustained doubling of AII levels by low salt diet.

Naomi *et al.* have shown in patients with essential hypertension that under high salt diet ($342\text{ mmol NaCl day}^{-1}$ for 7 days) mean Epo concentrations increased weakly by approximately 35% within the normal range, while haemoglobin concentrations decreased together with haematocrit [20]. In our study $100\text{ mg NaCl kg}^{-1}$ body weight were applied (which is the equivalent of $120\text{ mmol NaCl day}^{-1}$ for a 70-kg person). The 24-h urinary sodium excretion was of a similar order of magnitude for the low-salt group, while our high-sodium group clearly had a larger excretion. The minor increase of Epo concentration was ascribed by the authors to haemodilution due to water and salt retention. In the light of our findings their results are difficult to interpret and even more so as their study was done in patients with essential hypertension (but normal serum creatinine). Their actual medication was not given. In our experiments haematocrit and haemoglobin were well controlled and did not change clinically significantly. This excludes influences on the experiment by major changes of haematological parameters themselves.

Cole *et al.* have shown that anaemia is present in ACE knockout mice [36]. In the knockout mice, plasma Epo levels are only slightly elevated, but within the normal range (from $1.56 \pm 0.16\text{ mU ml}^{-1}$ to $2.71 \pm 0.29\text{ mU ml}^{-1}$). These data do not argue against an influence of AII on Epo secretion. They confirm, however, that AII is necessary together with Epo for the production of erythrocytes [37]. It may even be the case that the weak increase of Epo concentrations was due to the lack of AII. AII has been shown to induce the HIF1 transcription factor in cell cultures under normoxia [38]. Whether this is the case in humans during AII exposure is not known and will be difficult to test.

Taken together, our data show that under the chosen experimental setting in healthy subjects, endogenous AII does not appear to contribute significantly to the physiological regulation of Epo production. Our results in healthy subjects, however, do not preclude that in certain clinical conditions there may be an interplay of several factors which lead to a more pronounced effect of AII on the regulation of Epo, possibly on the local tissue level.

This work was supported by a grant (01 EC0001) from the German Bundesministerium für Bildung und Forschung. The authors would like to thank Mrs B. Proksch, Abteilung Klinische Pharmakologie, for expert technical assistance.

References

- 1 Fisher JW, Samuels AI, Langston JW. Effects of angiotensin and renal artery constriction on erythropoietin production. *J Pharmacol Exp Ther* 1967; **157**: 618–625.

- 2 Gould AB, Goodman S, DeWolf R, Onesti G, Swartz C. Interrelation of the renin system and erythropoietin in rats. *J Lab Clin Med* 1980; **96**: 523–534.
- 3 Onoyama K, Motomura K, Makita H, et al. Effects of long-term captopril on angiotensin II and erythropoietin levels and recovery from haemorrhagic anemia in rats. *Curr Ther Res* 1987; **41**: 472–479.
- 4 Gleiter CH, Freudenthaler S, Delabar U, et al. Erythropoietin production in healthy volunteers subjected to controlled haemorrhagia: evidence against a role of adenosine. *Br J Clin Pharmacol* 1996; **42**: 729–735.
- 5 Gleiter CH, Becker T, Wenzel J. Erythropoietin production in healthy volunteers subjected to controlled hypobaric hypoxia: further evidence against a role for adenosine. *Br J Clin Pharmacol* 1997; **44**: 203–205.
- 6 Gleiter CH, Becker T, Schreeb KH, Freudenthaler S, Gundert-Remy U. Fenoterol but not dobutamine increases erythropoietin production in humans. *Clin Pharmacol Ther* 1997; **61**: 669–676.
- 7 Freudenthaler SM, Schenck T, Lucht I, Gleiter CH. Fenoterol stimulates human erythropoietin production via activation of the renin angiotensin system. *Br J Clin Pharmacol* 1999; **48**: 631–634.
- 8 Pratt MC, Lewis-Barned NJ, Walker RJ, Bailey RR, Shand BI, Livesey J. Effect of angiotensin converting enzyme inhibitors on erythropoietin concentrations in healthy volunteers. *Br J Clin Pharmacol* 1992; **34**: 363–365.
- 9 Gleiter CH. Posttransplant erythrocytosis. A model for the investigation of the pharmacological control of renal erythropoietin production? *Int J Clin Pharmacol Ther* 1996; **34**: 489–492.
- 10 Torregrosa JV, Campistol JM, Montesinos M, Rogada AG, Oppenheimer F, Andreu J. Efficacy of captopril on posttransplant erythrocytosis. Long-term follow-up. *Transplantation* 1994; **58**: 311–314.
- 11 Plata R, Cornejo A, Arratia C et al. Angiotensin-converting-enzyme inhibition therapy in altitude polycythemia: a prospective randomised trial. *Lancet* 2002; **359**: 663–666.
- 12 Vlahakos DV, Kosmas EN. Losartan reduces haematocrit in patients with chronic obstructive pulmonary disease and secondary erythrocytosis. *Ann Intern Med* 2001; **134**: 426–427.
- 13 Donnelly S, Shah BR. Erythropoietin deficiency in hyporeninemia. *Am J Kidney Dis* 1999; **33**: 947–953.
- 14 Jensen JD, Eiskajer H, Madsen B, Egeblad M, Nielsen CB, Pedersen EB. Effect of captopril on the renal veno-arterial gradient of erythropoietin and oxygen in unilateral renal artery disease. *Scand J Clin Lab Invest* 1993; **53**: 859–865.
- 15 Julian BA, Gaston RS, Barker CV, Krystal G, Diethelm AG, Curtis JJ. Erythropoiesis after withdrawal of enalapril in posttransplant erythrocytosis. *Kidney Int* 1994; **46**: 1367–1403.
- 16 Urena P, Berehi L, Houhou S, et al. No acute change of serum erythropoietin in response to hypocalcemia or antihypertensive agents in uremic patients. *Nephron* 1995; **70**: 197–201.
- 17 Freudenthaler SM, Schreeb KH, Körner T, Gleiter CH. Angiotensin II increases erythropoietin production in healthy human volunteers. *Eur J Clin Invest* 1999; **29**: 816–823.
- 18 Freudenthaler SM, Lucht I, Schenck T, Brink M, Gleiter CH. Dose-dependent effect of angiotensin II on human erythropoietin production. *Eur J Physiol* 2000; **439**: 838–844.
- 19 Grossmann J, Burkardt R, Harder S, et al. Angiotensin II infusion increases plasma erythropoietin levels via an angiotensin II type receptor-dependent pathway. *Kidney Int* 2001; **60**: 83–86.
- 20 Naomi S, Umeda T, Iwaoka T. Endogenous erythropoietin and salt sensitivity of blood pressure in patients with essential hypertension. *Am J Hypertens* 1993; **6**: 15–20.
- 21 Klaassen RJ, van Gelder T, Rischen-Vos J, Deinum J, Man in't Veld AJ, Weimar W. Losartan, an angiotensin II receptor antagonist, reduces haematocrits in kidney transplant recipients with posttransplant erythrocytosis. *Transplantation* 1997; **64**: 780–782.
- 22 Wang AY, Yu AW, Lam CW, et al. Effects of losartan or enalapril on haemoglobin, circulating erythropoietin, and insulin-like growth factor-1 in patients with and without posttransplant erythrocytosis. *Am J Kidney Dis* 2002; **39**: 600–608.
- 23 Freudenthaler SM, Schreeb K-H, Wiese A, Pilz J, Gleiter CH. Influence of controlled hypoxia and radical scavenging agents on erythropoietin and malondialdehyde concentrations in humans. *Acta Physiol Scand* 2002; **174**: 231–235.
- 24 Eckardt KU, Bouteillier U, Kurtz A, et al. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 1989; **66**: 1785–1788.
- 25 Badzyska B, Grzelec-Mojzesowicz M, Dobrowolski L, Sadowski J. Differential effect of angiotensin II on blood circulation in the renal medulla and cortex of anaesthetised rats. *J Physiol* 2002; **538**: 159–166.
- 26 Eckardt KU, Kurtz A, Bauer C. Regulation of erythropoietin production is related to proximal tubular function. *Am J Physiol* 1989; **256**: F942–F947.
- 27 Gleiter CH, Brause M, Delabar U, Zebski H, Eckardt KU. Evidence against a major role of adenosine in oxygen-dependent regulation of erythropoietin in rats. *Kidney Int* 1997; **52**: 338–344.
- 28 Boer P, Koomans HA, Boer WH, Dorhout Mees EJ. Urinary angiotensin excretion during sodium restriction and diuretics. *Nephrol Dial Transplant* 1989; **4**: 713–717.
- 29 Boer P, Vos PF, Koomans HA, Rabelink AJ, Beutler JJ, Dorhout Mees EJ. Response of urinary angiotensin to challenges of the renin-angiotensin system. *Clin Chim Acta* 1991; **199**: 195–204.
- 30 Lall SB, Kunchaparty S, Siddiqui HH, Bajaj JS. Plasma renin activity and urinary kallikrein excretion in response to intravenous furosemide in diabetic patients. *Acta Diabetol* 1990; **27**: 337–342.
- 31 Wolff M, Jelkmann W. Clinical applicability of the determination of erythropoietin. In *Pathophysiology and pharmacology of erythropoietin*, eds Pagel H, Weiss CH, Jelkmann W. Berlin: Springer, 1992; 99–107.
- 32 Windhager E, ed. *Handbook of physiology*, Section 8, *Renal physiology*. Oxford: Oxford University Press, 1992.
- 33 Karlberg BE, Tolagen K. Relationships between blood pressure, age, plasma renin activity and electrolyte excretion in normotensive subjects. *Scand J Clin Lab Invest* 1977; **37**: 521–528.
- 34 Alexanian R. Correlations between erythropoietin production and excretion. *J Lab Clin Med* 1969; **74**: 614–622.
- 35 Schmid C, Castrop H, Reitbauer J, Della Bruna R, Kurtz A. Dietary salt intake modulates angiotensin II type 1 receptor gene expression. *Hypertension* 1997; **29**: 923–929.

- 36 Cole J, Ertoy D, Lin H, *et al.* Lack of angiotensin II-facilitated erythropoiesis causes anemia in angiotensin-converting enzyme-deficient mice. *J Clin Invest* 2000; **106**: 1391–1398.
- 37 Mrug M, Stopka T, Julian BA, Prchal JF, Prchal JT. Angiotensin II stimulates proliferation of normal early erythroid progenitors. *J Clin Invest* 1997; **100**: 3210–3214.
- 38 Richard DE, Berras E, Pouyssegur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1 in vascular smooth muscle cells. *J Biol Chem* 2000; **275**: 26765–26771.