Angiotensin II increases the release of endothelin-1 from human cultured endothelial cells but does not regulate its circulating levels

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ABSTRACT

We investigated the effect of angiotensin II on endothelin-1 secretion in vitro and in vivo. In vivo, angiotensin II was given intravenously to 23 essential hypertensive and 8 control subjects according to different protocols: Study A, 1.0 ng·min⁻¹·kg⁻¹ and 3.0 ng·min⁻¹·kg⁻¹ angiotensin II for 30 min each; Study B, 1.0 ng·min⁻¹·kg⁻¹ and 3.0 ng·min⁻¹·kg⁻¹ angiotensin II for 120 min each; Study C, 3.0 ng·min⁻¹·kg⁻¹ angiotensin II for 30 min followed by a dose increment of 3.0 ng·min⁻¹·kg⁻¹ every 30 min until mean blood pressure levels increased by 25 mmHg; Study D, 1.0 ng·min⁻¹·kg⁻¹ followed by 3.0 ng·min⁻¹·kg⁻¹ angiotensin II for 60 min each on two different NaCl diets (either 20 mmol NaCl/day or 220 mmol NaCl/day, both for 1 week). In all in vivo studies neither plasma nor urine endothelin-1 levels changed with angiotensin II infusion. In contrast, angiotensin II (10⁻⁹, 10⁻⁸, 10⁻⁷ mol/l) stimulated endothelin-1 secretion from cultured human vascular endothelial cells derived from umbilical cord veins in a time- and dose-dependent manner. The in vitro angiotensin II effects were abolished by candesartan cilexetil, an inhibitor of the membrane-bound AT₁ receptor, and also by actinomycin D, an RNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor, indicating that endothelin-1 release depended on AT₁ receptor subtype and de novo protein synthesis. Our findings indicate that angiotensin II regulates endothelin-1 release by cultured endothelial cells through an AT₁ receptor-dependent pathway, but does not influence circulating endothelin-1 levels in vivo.

INTRODUCTION

Human umbilical vascular endothelial cells (HUVECs) release both vasoconstrictor and vasodilator substances, thereby adjusting the local vascular tone [1–3].

Most potent among endothelium-derived vasoconstrictors is endothelin-1 (ET-1), a 21-amino-acid peptide originally isolated from porcine aortic cell cultures [4,5]. In vitro experiments showed a significant release of ET-1 by vascular endothelial cells after incubation with several substances, such as angiotensin II (ANG II) [6], adrenaline [1], arginine vasopressin (AVP) [7], transforming growth factor-β1 [8], interleukin-1 [9], thrombin [10,11], insulin-like growth factor-1 [12] and insulin [12].

Despite the above findings, while intravenous infusions of AVP [13] and insulin [12,14] significantly increased circulating ET-1 levels, pressor doses of ANG II did not affect urinary ET-1 levels in young female subjects [15]. Thus, the role of ANG II as a powerful controller of ET-1 release from the vascular endothelium in vivo remains questionable.

Key words: angiotensin II, endothelins, endothelium, hypertension.

Abbreviations: ALDO, aldosterone; ANG II, angiotensin II; AVP, arginine vasopressin; ET-1, endothelin-1; HUVECs, human umbilical vascular endothelial cells; PRA, plasma renin activity.

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Against this hypothesis, most filtered ET-1 is cleaved in the kidney by a neutral endopeptidase (EC 3.4.24.11) [16,17], which is abundant in the proximal tubule. Furthermore, the main source of renal ET-1 is the inner medullary collecting duct cells [18–20]. Thus, urinary ET-1 is likely to be of renal origin, and its measurement seems to be a bad indicator for evaluating the effects of ANG II on the vascular production of ET-1.

Klein et al. [15] also found unchanged plasma ET-1 levels after infusion of ANG II for 30 and 60 min. As correctly pointed out [15], this finding does not allow speculation regarding the effects of ANG II on ET-1 release by the endothelial lining of blood vessels. Indeed, the production of significant amounts of ET-1 by human vascular endothelial cells requires 2–3 h (reviewed in [21]), even when ANG II is used as secretagogue [6,7,21]. Consequently, the relationship between circulating ANG II and ET-1 in vivo remains to be evaluated.

To address this topic, we examined the effects of short-term and prolonged ANG II infusions, at different doses, on circulating and urinary ET-1 concentrations. To eliminate the possible influence of inter-individual variability in blood pressure levels and responses to ANG II, we decided to conduct all experiments in both normal and hypertensive individuals and to test the effects of equipressor doses of ANG II. Since sodium intake may influence the vascular responsiveness to ANG II [22–24], the latter was also re-infused after a further week on either a low or a high sodium diet.

Apart from the in vivo experiments, since the ANG II effect on ET-1 release has been evaluated previously only in rat mesenteric [6] and bovine carotid endothelial cells [7], we also examined the effect of ANG II on ET-1 release by cultured human vascular endothelial cells (HUVECs). In this regard, about 75% of spontaneous ET-1 release is directed towards the basolateral compartment of HUVECs [25], suggesting that in vivo ET-1 secretion is mostly directed towards the underlying vascular smooth muscle cells but not secreted into the bloodstream. Therefore, we also evaluated whether ANG II may interfere with the polarity of ET-1 secretion by cultured HUVECs.

**METHODS**

**In vitro studies**

Experiments with HUVECs were conducted as described previously for the determination of insulin-mediated ET-1 release, with slight modifications [12]. Briefly, HUVECs were isolated according to the method of Jaffe et al. [26] and then grown to confluence in 24-well dishes in Dulbecco’s HAT medium, supplemented with 5% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 °C in humidified 5% CO2 in air. After incubation for 1 week, confluent cells were replaced in 35-mm dishes containing serum-free Dulbecco’s HAT medium. HUVECs were then incubated for various times up to 24 h either alone or with ANG II, 10^{-3}–10^{-7} mol/l (Sigma Chemical Co., St Louis, MO, U.S.A.), or equimolar doses of the test substances human recombinant insulin (Ely-Lilly Italia Spa, Florence, Italy) and AVP (Sigma).

In subsequent experiments, HUVECs were incubated with either 10^{-6} mol/l actinomycin D (Sigma), an RNA synthesis inhibitor, added alone or concomitantly with ANG II, or 10^{-6} mol/l cycloheximide (Sigma), a protein synthesis inhibitor, added alone or 180 min before ANG II, or 10^{-4} mol/l candesartan cilexetil (kindly donated by Takeda Italia Farmaceutici SpA, Rome, Italy), an inhibitor of the membrane-bound AT1 receptor, added alone or 30 min before ANG II.

**Effects of ANG II on the polarity of ET-1 secretion**

For this study, HUVECs were cultured by the method of Wagner et al. [25], with slight modifications. In brief, HUVECs were grown to confluence as described above and then, after a further three passages in serum-free Dulbecco’s HAT medium, seeded at high density on to acellular collagen matrix, comprising bovine type I and type III collagen (Sigma). The stromal surface was suspended on a Teflon substrate (porosity: 0.4 µm) which was placed into tissue culture wells. After confluence, the resulting endothelial monolayer divided into two compartments, an upper one (2.5 ml) on the luminal side, and a lower one (2.5 ml) on the basolateral side. Thereafter, cells were incubated alone or with either ANG II (10^{-3}–10^{-7} mol/l) or equimolar doses of the test substances AVP and insulin, for various time up to 24 h.

**ET-1 assay**

The peptide was assayed as described previously [12]. In brief, reverse-phase HPLC was used to characterize the secretion of ET-1. For this purpose, secretion media from four 100-mm plates were centrifuged at 15 000 g for 10 min. Each mixed supernatant was subsequently freeze-dried and reconstituted in starting HPLC buffer. After injection on to C18 octylsilane columns (Pharmacia, Uppsala, Sweden) equilibrated with 0.1% trifluoroacetic acid, samples were eluted over 70 min using a linear gradient of 15–75% acetonitrile/0.1% trifluoroacetic acid in water. Fractions were collected every minute and evaporated before reconstitution in assay buffer (50 mmol/l phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.05% NaN3 and 0.5% BSA). ET-1 immunoreactivity was then assayed on reconstituted samples by a sensitive radioimmunoassay (Peninsula Laboratories, Belmont, CA, U.S.A.). Human ET-1 (Peptide Institute, Osaka, Japan) was used as standard. Inter- and intra-assay variations were < 10%. Mean recovery of the complete procedure, i.e. from HPLC purification...
to radioimmunoassay, was 82%. Cross-reactivity of the ET-1 antibody with Big ET-1 and with ET-2 and ET-3 was <16% and 7% respectively, according to the supplier.

**In vivo studies**

*In vivo* protocols were approved by our Ethics Committee. Informed consent to take part in the study was requested from normal volunteers and eligible hypertensive subjects, who were selected from consecutive outpatients aged between 35 and 60 years.

With regard to hypertensive individuals, we selected only outpatients with a supine diastolic blood pressure between 95 and 114 mmHg after 1 week of pharmacological washout and then at four different visits performed at 1-week intervals. In order to exclude the bias of Type 2 diabetes [27], obesity [28], hyperlipidaemia [29] and atherosclerosis [30], and of renal [31], liver [32] or cardiac [33] diseases (i.e. of all known conditions that may potentially alter circulating ET-1 levels), during the above 4-week period the following inclusion criteria were verified: body mass index >19 and <27 kg/m², serum creatinine <110 mmol/l; normality of $^{99m}$Tc-diethylenetriaminepenta-acid scintirenogram, absence of proteinuria and microalbuminuria <20 µg/min in three different 24-h collections, normal levels of aspartate aminotransferase and alanine aminotransferase. A normal glucose tolerance was proved by the presence of fasting glucose levels <6.0 mmol/l, absence of glycosuria, and a plasma glucose response to oral glucose load (75 g) that was <10.0 mmol/l. Patients were also required to present normal serum cholesterol and triacylglycerol levels (i.e. serum cholesterol <5.2 mmol/l and >3.9 mmol/l and serum triacylglycerols <1.7 mmol/l and >1.1 mmol/l). The normality of cardiovascular apparatus was assessed by clinical evaluation, one- and two-dimensional echocardiograms, and 12-lead ECG. The absence of atherosclerotic lesions of the neck and limb vessels was proved by clinical evaluation, one- and two-dimensional echo-Doppler examinations. Hyperensive retinopathy was required to be below grade II in all cases. Patients with a personal history of ischaemic brain, cardiac and/or leg disease, as well as those with either alcohol or smoking behaviour, were excluded from the study. The secondary forms of hypertension were screened out by clinical and laboratory assessments.

After drug withdrawal, each patient was assigned to a weight-maintaining diet with a constant sodium intake (120 mmol NaCl daily). Each patient followed the diet for the 4-week period necessary for the evaluation of inclusion criteria. The normal sodium diet was achieved by a daily supplement of 10 capsules (each capsule containing 10 mmol NaCl) added to a diet containing about 1 g/kg proteins, 2 g/kg carbohydrates, 0.5 g/kg fats, 20 mmol Na⁺ and 60 mmol K⁺ per day. As described previously [34,35], participants were interviewed about dietary habits, and then carefully instructed by us and experienced nutritionists on how to avoid any kind of high sodium food, to appreciate low sodium food, and to completely eliminate added salt. ‘Tailored’ dietary written advice was also given to all patients, and medical staff were available from 08:00 to 20:00 hours to answer any queries about diet. To simulate as closely as possible the Italian pattern of eating, the capsules were administered three times a day, i.e. during breakfast, lunch and dinner. Patients were also advised to drink 2.0 litres of tap water per day. Adherence to the diet was assessed by measuring the 24-h urinary sodium and chloride excretions on the last 3 days of each week. Patients were considered compliant when sodium and chloride excretions were >80 mmol/day and <130 mmol/day.

After evaluation of the above criteria and 4 weeks on the diet of 120 mmol NaCl/day, 23 patients (12 males and 11 females, mean age 46.5 ± 0.9 years) were definitively enrolled in the study. Each of these hypertensive subjects was then assigned to three study sessions, to be performed on separate days at 1-week intervals during a 3-week study period.

All the *in vivo* experiments were also performed in eight age-matched normotensive volunteers (five males and three females, mean age 45.4 ± 1.1 years). Normotension was defined as a blood pressure constantly <130/85 mmHg. Procedures and inclusion criteria were identical to those used for hypertensive subjects.

**Procedures**

For the first *in vivo* experiment (Study A), both patients and controls continued the assigned diet of 120 mmol NaCl/day. At 08:00 hours, after an overnight fast, each subject brought a 24-h urine collection and was admitted to our outpatient unit. Two intravenous lines were inserted in the forearm veins for withdrawal of blood and infusions respectively. After 1 h in the supine position, either ANG II dissolved in 50 ml of isotonic saline ($n = 9$ patients) (1.0 and 3.0 ng·min⁻¹·kg⁻¹ for 30 min each) or placebo ($n = 6$ patients) (50 ml of isotonic saline) were infused, according to a randomized, double-blind protocol. Blood samples for plasma renin activity (PRA), aldosterone (ALDO) and ET-1 measurements were taken every 15 min during the ANG II infusion, and then every 30 min for a further 2 h. Patients were asked to void before (to complete the 24-h urine collection) and at the end of both ANG II and placebo infusions, and also 2 h later. Four control subjects (three males and one female) participated in Study A. None of the control subjects was infused with placebo.

In a subset of randomly selected patients ($n = 5$) who participated in Study A, both the 1.0 and 3.0 ng·min⁻¹·kg⁻¹ ANG II infusions were continued for 120 min each (Study B). Three patients were infused with placebo for 4 h. Venous blood samples for PRA, ALDO and ET-1 measurements were taken every 30 min during
the infusion and then after 2 h recovery. Arterial blood samples for ET-1 measurements were also drawn in these patients, by using the contralateral brachial artery and an intra-arterial catheter. Arterial blood was collected at baseline, and then every 30 min during the ANG II infusion and after 2 h recovery. Urine collections for ET-1 assay were made at the beginning and end of both ANG II and placebo infusions, and 2 h later. Throughout the infusion, in both the above studies, blood pressure was constantly measured every 10 min by a standard Riva-Rocci sphygmomanometer and a stethoscope. Four control subjects (two males and two females) participated in this study. None of them was infused with placebo.

One week after this experiment, patients were re-infused with either placebo (n = 14 patients) or ANG II (3.0 ng·min⁻¹·kg⁻¹ for 30 min) (n = 9 patients) (Study C). The ANG II dose was augmented by 3.0 ng·min⁻¹·kg⁻¹ every 30 min, until mean blood pressure levels increased by 25 mmHg for three determinations at 10-min intervals. Blood samples for PRA, ALDO and ET-1 measurements were taken every 30 min during both ANG II and placebo infusions, and then every 60 min for a further 2 h. Urine collections for ET-1 measurements were made in all patients at the beginning and end of infusions, and then after 2 h recovery. All of the control population participated in this study.

For the last in vitro experiment (Study D), either ANG II (1.0 ng·min⁻¹·kg⁻¹ and 3.0 ng·min⁻¹·kg⁻¹ for 60 min each) or placebo were randomly infused as in the first protocol, but after dietary NaCl intake was changed according to a randomized, double-blind protocol. For this purpose, 12 patients were assigned to a low sodium diet (20 mmol NaCl/day for 1 week) and 11 patients to a high sodium diet (220 mmol NaCl/day for 1 week). Both the low and the high sodium intakes were achieved by continuing the previous diet, but substituting the daily supplement of 10 capsules containing 10 mmol NaCl each with identical capsules containing either placebo (meal) or 20 mmol NaCl each. Compliance was verified by measuring both sodium and chloride excretions on the last 3 days of the week. Three patients with a 24-h urinary excretion of sodium > 20 mmol/day during the low sodium intake and one patient with a sodium excretion < 180 mmol NaCl during the high sodium intake were considered not compliant and excluded from the study. Thus, the last experiment was performed in only 19 patients. In the low sodium group, five patients were infused with ANG II and four with placebo. In the high sodium group, six patients were infused with ANG II and the remaining four received placebo. In Study D, on both diets, urine samples for ET-1 assay and blood samples for PRA, ALDO and ET-1 assays were collected before, during and after the ANG II infusion, as already explained for Study A. Four control subjects (three males and one female) were assigned to the low sodium diet, and the remaining four (two males and two females) received the high sodium diet. None of them was infused with placebo.

For studies C and D, blood pressure was measured every 10 min by a standard Riva-Rocci sphygmomanometer and a stethoscope.

**Laboratory measurements**

Plasma and urinary ET-1 levels were assessed as for cell supernatants, with slight modifications, as described previously [12,27,28]. PRA and ALDO concentrations were assayed by commercial RIA kits (Sorin Biomedica, Saluggia, Italy). The other routine laboratory tests were performed by standard laboratory methods.

**Statistical analysis**

For in vitro experiments, analysis of variance followed by the appropriate post hoc test (Student/Newman–Keuls’ test) was used for comparisons between different ANG II concentrations as well as for comparisons between ANG II and other substances at each time point. Repeated measures were evaluated by analysis of variance followed by Bonferroni’s test for both in vivo and in vitro experiments. Statistical significance was considered for a P value < 0.05. Unless otherwise stated, data are presented as means ± S.E.M. In the case of in vitro experiments, each value represents the mean ± S.E.M. from four replicate determinations in four different experiments.

**RESULTS**

**In vitro studies**

ANG II significantly stimulated ET-1 release from cultured HUVECs in a time- and dose-dependent manner (Figure 1). ANG II, 10⁻⁸ mol/l, was more potent

![Figure 1](https://example.com/figure1.png)

**Figure 1** ANG II and endothelin-1 secretion

Endothelin-1 secretion from confluent vascular endothelial cells derived from human umbilical cord vein over 24 h both in the absence (open bars) and in the presence of different concentrations of ANG II ([■], 10⁻⁹ mol/l; [□], 10⁻⁸ mol/l; [▲], 10⁻⁷ mol/l). Each bar represents the mean ± S.E.M. of four determinations from four experiments. *P < 0.05 versus control; †P < 0.05 versus ANG II, 10⁻⁹ mol/l; #P < 0.05 versus ANG II 10⁻⁸ mol/l and 10⁻⁷ mol/l.
than insulin, 10⁻⁹ mol/l (P < 0.01 after 24 h incubation), and, less significantly, AVP, 10⁻⁹ mol/l (P < 0.01 after 24 h incubation), in promoting ET-1 release from cultured HUVECs.

Cell incubation with actinomycin D (10⁻⁶ mol/l) both in the presence and absence of ANG II resulted in a complete blockade of spontaneous and ANG II-stimulated ET-1 release from cultured cells, suggesting that de novo protein synthesis is necessary for ET-1 secretion. Accordingly, cycloheximide (10⁻⁵ mol/l), a protein synthesis inhibitor, significantly reduced spontaneous and ANG II-stimulated ET-1 release. Selective inhibition of the AT₁ receptor subtype by candesartan cilexetil (10⁻⁶ mol/l) did not affect the spontaneous secretion of ET-1 but completely abolished the ET-1 response to ANG II.

According to previous data [25], ET-1 secretion was modified by ANG II administration (Figure 3A). Similar findings were obtained for urinary ET-1 levels (Figure 3B).

**In vivo studies**

**Study A**

As already reported [34,35], infusion of ANG II (1.0 ng min⁻¹·kg⁻¹ and 3.0 ng min⁻¹·kg⁻¹ for 30 min each) in essential hypertensive subjects produced minor blood pressure changes (systolic: from 160.56 ± 1.54 mmHg at time 0 to 163.52 ± 1.94 mmHg after 60 min, P = 0.228; diastolic: from 100.51 ± 1.03 mmHg at time 0 to 102.51 ± 1.03 mmHg after 60 min, P = 0.356), whereas PRA was significantly reduced (from 2.39 ± 0.22 pmol ANG I·h⁻¹·ml⁻¹ at time 0 to 1.08 ± 0.14 pmol ANG I·h⁻¹·ml⁻¹ after 60 min, P < 0.0001) and plasma ALDO concentrations were increased (from 356.5 ± 62.87 pmol/l at time 0 to 772.64 ± 98.12 pmol/l at 60 min, P = 0.003). The ANG II infusion also significantly reduced urinary sodium excretion (from 0.08 ± 0.01 mmol/min at baseline to 0.02 ± 0.11 mmol/min after 60 min, P < 0.0001), while a non-significant reduction of urinary volume was observed at the end of infusion (from 1.01 ± 0.13 ml/min to 0.80 ± 0.11 after 60 min, P = 0.116). In spite of these endocrine and renal changes, plasma ET-1 levels were not significantly modified by ANG II administration (Figure 3A). Similar findings were obtained for urinary ET-1 levels (Figure 3B).
Figure 4 Effects of placebo (50 ml of isotonic saline) or ANG II infusions (4 h) on plasma (A) and urinary endothelin-1 concentrations (B) in essential hypertensive patients (HYP) and normal volunteers (CON).

In (A) the S.E.M. has been omitted for clarity, and the thick black line represents the duration of ANG II infusion.

Study B
In the hypertensive subset who received a 4-h infusion of ANG II (n = 5), both renal and endocrine changes were particularly evident (PRA: from 2.60 ± 0.19 pmol ANG I h⁻¹ ml⁻¹ at baseline to 0.69 ± 0.03 pmol ANG I h⁻¹ ml⁻¹ after 4 h, P < 0.0001; ALDO: from 403.96 ± 67.57 pmol/l at baseline to 1643.64 ± 246.37 pmol/l after 4 h, P < 0.001; urinary sodium: from 0.09 ± 0.01 mmol/min at baseline to 0.02 ± 0.01 mmol/min after 4 h, P < 0.0001). In addition, the pressor response to ANG II became more evident with the 4-h infusion, and significant increments in diastolic blood pressure levels were observed at the end of ANG II administration (from 102.36 ± 0.89 mmHg at baseline to 112.54 ± 2.01 mmHg after 4 h, P < 0.002). In spite of these findings, both venous (Figure 4A) and urinary ET-1 levels (Figure 4B) failed to increase with ANG II infusion. With regard to arterial ET-1, levels were lower than for venous ET-1, with an arterial-to-venous ratio that was less than unity in all cases (0.79 ± 0.02 in hypertensive subjects and 0.81 ± 0.02 in normotensive controls, P not significant). As with venous ET-1, ANG II infusion failed to increase levels of arterial ET-1 (hypertensive subjects: from 2.05 ± 0.10 pg/ml at baseline to 2.13 ± 0.14 pg/ml at the end of infusion; normotensive subjects: from 2.04 ± 0.08 pg/ml at baseline to 2.10 ± 0.13 pg/ml at the end of infusion). As a consequence, the mean arterial-to-venous ratio remained unchanged in both groups during the ANG II infusion as well as after the 2-h recovery.

Study C
When ANG II was infused at equipressor doses, the amount of ANG II which was necessary to increase mean blood pressure by 25 mmHg ranged from 6 ng min⁻¹ kg⁻¹ (in four hypertensive patients) to 21 ng min⁻¹ kg⁻¹ (in one hypertensive patient) (mean 11.0 ± 5.8 ng min⁻¹ kg⁻¹). Consequently, the infusion time ranged from 1 to 3.5 h (mean 1.81 ± 0.94 h). Despite the greatest increments in plasma ALDO levels (from 201.52 ± 34.21 pmol/l to 864.54 ± 67.87 pmol/l, P < 0.0001) and decrements in PRA (from 2.24 ± 0.30 pmol ANG I h⁻¹ ml⁻¹ to 0.33 ± 0.03 pmol ANG I h⁻¹ ml⁻¹, P < 0.0001) occurring in Study C, neither plasma (Figure 5A) nor urinary ET-1 levels (Figure 5B) changed significantly during and/or after the ANG II infusion. Similar findings were obtained in control subjects, who received ANG II doses ranging from 6 to 18 ng min⁻¹ kg⁻¹ (mean 9.8 ± 4.4 ng min⁻¹ kg⁻¹) (Figure 5).

Study D
As expected [34,35], changes in dietary NaCl intake altered the adrenal and PRA responses to infused ANG II. Indeed, PRA levels were suppressed by the high NaCl
increased baseline PRA (\(P < 0.0001\)) and ALDO levels (\(P < 0.0001\)). Similarly, the mean ALDO increment with ANG II infusion was particularly evident when the ANG II infusion was performed on a low NaCl intake. In any case, the absence of significant increments of plasma and urinary ET-1 in both hypertensive and normotensive subjects after ANG II infusion seems unsound. Even more perplexing, ET-1 secretion from HUVECs is mainly directed towards the basolateral side [25] and, as already indicated by our group for other substances [37], demonstrates that neither ANG II, nor insulin, nor AVP were able to affect the polarized secretion of ET-1.

In agreement with previous in vitro findings, ANG II, at equimolar doses, was a more potent stimulator of ET-1 secretion by endothelial cells than insulin [38] and, less significantly, AVP [7]. Since AVP [13] and insulin [12,14,39] have been reported to induce mild but significant increments of plasma and urinary ET-1 in both animals [13,39] and humans [12,14], even when infused intravenously for 1 or 2 h [12–14], a marked ET-1 increase was expected with an ANG II infusion in vivo. Therefore, the observed absence of significant changes in ET-1 levels in plasma and urine from essential hypertensive and normotensive subjects after ANG II infusion seems unsound. Even more perplexing, ET-1 changes were not observable after either short-term infusion of subpressor or prolonged administration of equipressor doses of ANG II (range 6.0–21.0 ng \(\text{min}^{-1} \cdot \text{kg}^{-1}\), mean \(11.0 \pm 5.8 \text{ng} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}\)). Furthermore, no changes in venous, arterial or urinary ET-1 levels were obtained in hypertensive patients receiving a 4-h ANG II infusion, or when ANG II was re-infused after either a low or a high sodium diet.
In this context, the results observed in normotensive subjects confirmed the absence of ET-1 modifications during ANG II infusions, and excluded that it was dependent on hypertension-related vascular damage. Thus, we think that our results clearly demonstrate that circulating ANG II does not affect venous, arterial and urinary ET-1 concentrations, regardless of the rate and duration of ANG II infusion, blood pressure levels at baseline and after infusion, and sodium intake.

The reasons leading to the above in vitro–in vivo discrepancies are unclear. With regard to possible study bias, we carefully eliminated all conditions that might alter ET-1 production, such as diabetes [27], obesity [28], hyperdyslipidaemia [29], atherosclerosis [30], and renal [31], liver [32] and cardiac diseases [33]. Experiments were placebo-controlled and all the in vivo studies were conducted according to a randomized protocol, in control and patient groups that were sufficiently large for statistical evaluation. Thus, neither confounding factors nor type I and type II statistical errors [40] should have influenced our results. Similarly, repeated blood and urine samples for ET-1 assay were obtained from patients during ANG II infusions, which lasted from 1 to 4 h, as well as after 2-h recovery. Since HUVECs secrete significant amount of ET-1 within 2–3 h (reviewed in [21]), the duration of our in vivo experiments, at least for Studies B and C, was sufficient to detect eventual ANG II-related ET-1 increments in both plasma and urine. In this regard, we infused ANG II intravenously, and measured ET-1 in venous plasma in 3 of 4 in vivo experiments. Thus, a significant ANG II-induced ET-1 increase might occur in arterial plasma and, due to the polarity of ET-1 secretion by the vascular endothelium and ET-1 degradation by the pulmonary vasculature [41], be undetectable in venous plasma. Against this possible study limitation, Study B confirmed that baseline arterial ET-1 concentrations were lower than venous concentrations, with an arterial-to-venous ratio below unity in both hypertensive and normotensive subjects. Although obtained in small subsets of hypertensive (n = 5) and normotensive subjects (n = 4), we found no significant changes in arterial ET-1 levels and arterial-to-venous ET-1 ratio after a 4-h ANG II infusion. On the other hand, human venous endothelial cells are able to produce significant amounts of ET-1 [42], whereas two reports did not confirm the role of the pulmonary vasculature in ET-1 removal [13,43]. Furthermore, in agreement with the results obtained in Study B, no changes in either venous or arterial ET-1 levels were observed in conscious dogs who were infused intravenously with ANG II [13]. Interestingly, the dogs received ANG II at doses that were reminiscent of those used in our protocols, i.e. 2 ng·min⁻¹·kg⁻¹ and 5 ng·min⁻¹·kg⁻¹ for 60 min each. In the same experimental model, intravenous infusion of higher doses of ANG II, i.e. 10 ng·min⁻¹·kg⁻¹, also failed to increase ET-1 levels, whereas equivalent doses of AVP induced a significant rise in venous ET-1 concentrations [13]. Consequently, we think that the lack of ET-1 changes that we observed after intravenous ANG II infusion clearly reflects the lack of effects of infused ANG II on circulating ET-1. Obviously, it is extremely likely that a significant amount of ET-1 could have been produced and secreted by vascular endothelial cells towards the basolateral membrane after ANG II stimulation, but we have no data to support such a hypothesis.

In the current study, we also showed that urinary ET-1 excretion remained unchanged after ANG II infusions. This finding is in accordance with those obtained by Klein et al. [15], who observed that phenylephrine but not ANG II was able to increase urinary ET-1 excretion in humans. In this regard, it is well known that most filtered ET-1 is subject to degradation by neutral endopeptidase in the proximal tubule [16,17]. Accordingly, less than 0.3% of intravenously administered ¹²⁵I-ET-1 appears in urine [16,17]. Thus, urinary ET-1 is generally believed to be of renal origin. In particular, although vascular endothelial, mesangial and epithelial cells produce significant amounts of ET-1 [17,44] and ET-1 mRNA [18], the main source of renal ET-1 seems to be the inner medullary collecting duct cells [17]. In this segment, locally produced ET-1 acts as an autocrine–paracrine agent in the tubular lumen [45,46]. Therefore, urinary ET-1 should be taken as an indicator of the renal paracrine ET-1-forming system rather than the vascular system.

As already mentioned, both plasma and urinary ET-1 levels might not reflect the in vivo interrelations between ANG II and ET-1. Moreover, there are several aspects of vascular physiology that could have determined, or contributed to, the observed in vivo–in vitro discrepancy. The first is the polarization of ANG II-stimulated ET-1 secretion, which can make plasma ET-1 measurement an unreliable method for evaluating endothelial ET-1 production. In addition, the vascular endothelial cell expresses an enzyme that degrades ET-1 and prevents peptide secretion into the circulation [47]. Furthermore, vascular smooth muscle cells produce a soluble unknown inhibitor(s) of ET-1 secretion by endothelial cells [48]. Finally, as suggested for coronary vascular endothelial cells in vitro, some vascular districts may be insensitive to ANG II, at least as far as ET-1 secretion is concerned [49]. Therefore, the absence of plasma venous and urinary ET-1 changes after stimulation with ANG II might be due to a constellation of factors. All of the latter cannot be fully evaluated in vitro, and the results obtained in cell cultures cannot be transposed sic et simpliciter to in vivo settings. In particular, it should be emphasized that HUVECs are not representative of the behaviour of living adult endothelial cells.

In this context, a last comment must be addressed to two recent studies [50,51] that apparently contrast with
our findings. In the first report, Balakrishnan et al. [50] elegantly demonstrated that bosentan, a non-selective ET-1 antagonist, reduced the pressor response to ANG II in rats, therefore suggesting that ANG II stimulates ET-1 production in vitro. In the second report, Jilma et al. [51] suggested that ANG II, infused at doses similar to those used in our Study C, significantly increased plasma ET-1 levels in a serial group of healthy volunteers. We do not think that the study by Balakrishnan et al. [50] contradicts our findings. As discussed above, the lack of increase in plasma ET-1 during ANG II infusion does not imply that ET-1 has not been simultaneously secreted towards the basolateral side of vascular endothelial cells. The discrepancy between our results and those of Jilma et al. [51] can be explained by the lack of HPLC extraction before ET-1 measurement, which is known to lead to overestimation of the ET-1 concentration [12,37], and by the use of multiple venepunctures during ANG II infusion, which are known to induce local ET-1 release [52].

In conclusion, this study demonstrated that treatment of HUVECs with ANG II resulted in an increased release of ET-1 in vitro, i.e. a selective inhibitor of AT1 receptor, candesartan cilexetil, completely blocked the ANG II action. In this regard, we also showed that ANG II was more potent than AVP and insulin, i.e. two well-recognized stimulators of ET-1 release in vitro. None of these agents was able to modify the polarity of ET-1 secretion by cultured cells.

In spite of the above findings, intravenous administration of ANG II to essential hypertensive subjects and healthy volunteers did not lead to an increase in plasma and urinary ET-1 levels. The lack of ANG II effects on ET-1 did not depend on the dose of ANG II, duration of infusion or sodium intake. A study limitation could be the use of multiple venepunctures during ANG II infusion, which are known to induce local ET-1 release [52].

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