Plasma Endothelin Is Increased in Early Essential Hypertension

Markus P. Schneider, Karl F. Hilgers, Arnfried U. Klingbeil, Stefan John, Roland Veelken, and Roland E. Schmieder

Local vascular generation of endothelin-1 (ET-1) may contribute to elevated peripheral resistance in hypertension. We tested the hypothesis that immunoreactive ET production in the forearm circulation is increased in early essential hypertensive subjects. Ten young, previously untreated male patients with mild essential hypertension and no signs of target organ damage were compared with matched normotensive subjects in an outpatient setting. Arterial and venous samples were obtained from indwelling catheters in the brachial artery and the medial cubital vein, respectively. Samples were collected at baseline and after induction of endothelium-dependent (acetylcholine) vasodilation. Immunoreactive ET (ET) was measured after column extraction by a sensitive radioimmunoassay employing a C-terminal ET-1 antibody with negligible cross-reaction to big-ET. Individual recovery rates were determined for each sample.

Basal ET was significantly higher in hypertensive than in normotensive subjects, both in venous and arterial samples ($P < .01$). This difference was also present after correction for recovery ($P < .01$). There was no significant difference between venous and arterial ET concentrations. Local vasodilation did not change arterial or venous ET levels. In conclusion, plasma ET is increased in young, untreated, essential hypertensive subjects with no signs of target organ damage. The increased circulating immunoreactive ET may point to a role for the peptide early in the development of high blood pressure. Am J Hypertens 2000;13:579–585 © 2000 American Journal of Hypertension, Ltd.

KEY WORDS: Endothelin, forearm, hypertension, peripheral resistance.
The role of ET in the pathogenesis of hypertension. To eliminate these confounding factors, young untreated hypertensives without any signs of end-organ damage need to be studied.

Endothelin most likely acts in a paracrine fashion, and venous plasma levels of ET may differ in various vascular beds. Thus, arterial blood levels may be more representative than venous blood levels. Furthermore, it seems helpful to simultaneously determine arterial and venous plasma levels for one vascular bed to gain information about the local vascular ET formation.

To test the hypothesis that ET-1 formation is increased at the onset of essential hypertension we measured immunoreactive ET blood levels in young untreated hypertensive patients using an assay that does not cross-react with big-ET-1. Brachial artery and forearm venous blood samples were obtained simultaneously to detect local vascular ET formation. Because we hypothesized that ET-1 formation might be stimulated by vasodilation, samples were also collected after intraarterial infusion of endothelium-dependent (acetylcholine) vasodilators.

**MATERIALS AND METHODS**

**Study Cohort** The study population comprised 10 young male white subjects with mild to moderate essential hypertension, World Health Organization (WHO) stage I, and 10 young male healthy subjects with normal blood pressure (for patient characteristics, see Table 1). Hypertensive patients and normotensive controls were recruited by screening students for high blood pressure at the university campus. Casual blood pressure readings were performed in a sitting position after 5 min of rest with a mercury sphygmomanometer according to the recommendations of the WHO. Normotension was defined by blood pressure values below 140 mm Hg systolic and below 90 mm Hg diastolic at all blood pressure measurements. Inclusion criteria for the hypertensive group was that the average of the four blood pressure readings had to exceed 160/95 mm Hg but had to be below 200/115 mm Hg. Ambulatory blood pressure was monitored by an automatic, noninvasive device (SpaceLabs 90207, SpaceLabs, Redmond, WA) and average 24-h blood pressure had to be greater than 130 mm Hg systolic or greater than 80 mm Hg diastolic to be included in the study. None of the patients had ever received cardiovascular medication in the past. None of the patients followed any specific dietary guidelines before the invasive evaluation. Normotensive subjects participated as volunteers and underwent a complete routine clinical work-up to ensure that their cardiovascular system was normal. Patients with essential hypertension were enrolled only if secondary hypertension, as well as WHO stage II or III hypertensive disease, had been ruled out. Therefore, exclusion criteria were hypertensive fundoscopic changes, any evidence of coronary, valvular, or myocardial disease (in particular, myocardial infarction or congestive heart failure), microalbuminuria, or renal insufficiency. Exercise stress testing or detailed evaluation of renal arteries (intraarterial digital subtraction angiography) and hormone measurements were conducted only if clinically indicated. Informed written consent was obtained from each individual. The protocol was approved by the local University Investigation Ethics Committee.

**Study Protocol** The morning of the experiment, the participant lay in a quiet laboratory room in the supine position. At the beginning, catheters in the brachial artery and cubital vein were inserted. Blood samples were drawn from the brachial artery and cubital vein without suction at the same time of the day for each participant. For basal levels, the participant had been supine for at least 30 min. After another resting period of 30 min acetylcholine was infused at increasing doses (3, 12, 48, 192 μg/min) intraarterially for 3 min each. The flow rate for all infusions was held constant at 1.5 mL/min. The internal diameter of the

<table>
<thead>
<tr>
<th>TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION (MEAN ± SEM)</th>
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<tr>
<td><strong>Normotensive Subjects</strong></td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Height (m)</td>
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<tr>
<td>Weight (kg)</td>
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<tr>
<td>Body mass index (kg/m²)</td>
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<tr>
<td>Ambulatory systolic BP (mm Hg)</td>
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<tr>
<td>Ambulatory diastolic BP (mm Hg)</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dL)</td>
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<td>Serum triglycerides (mg/dL)</td>
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NS = not significant.
radial artery was continuously registered throughout the experiment by applying high-resolution ultrasonic techniques (NIUS 02, Asulab Paris, France; for details, see References 18, 19). Immediately at the end of the infusion period arterial and venous blood samples were drawn again.

Measurement of Endothelin  All blood samples were collected in tubes containing chilled potassium EDTA and immediately centrifuged at 4°C. The plasma was separated and frozen at −70°C until the assay. In pilot experiments, four different cartridge extraction methods were tested: A) Amprep C2 cartridge, 500 mg, (Amersham International, plc, Aylesbury, Bucks, UK) were equilibrated by washing with 2 mL methanol followed by water; 1 mL plasma was acidified with 0.25 mL 2 mol/L HCl, centrifuged for 5 min at room temperature, and loaded onto the column. The column was washed with 5 mL water +0.1 trifluoracetic acid (TFA), then eluted with 2 mL 80% methanol in water +0.1% TFA and the eluate collected in polypropylene tubes. The eluate was dried in a centrifugal evaporator, redissolved in 250 μL assay buffer (0.02 mol/L borate buffer, pH 7.4, containing 0.1% sodium azide), and 2 × 100 μL was taken for analysis; B) same procedure as (A), but with Bond-Elut C18 (Varian, Harbor City, CA) cartridges; C) same procedure as (A), but with SepPak Vac/3ccC18 (Waters, Milford, MA), additionally loading columns with 1 mL 1% polypeptide solution and washing with 2 mL 80% methanol in water before loading columns with plasma; and D) this method was later used for measuring ET immunoreactivity in the plasma of the patients. Plasma was extracted using Sep Pak Vac/3cc C18 cartridges. The cartridges were conditioned with 2 mL methanol followed by 2 mL of 0.2 mol/L phosphate/citric acid, pH 7.0; 0.5 mL of plasma were passed through equilibrated cartridges at low speed (1 mL/min). After washing with 2 mL of water, the cartridges were eluted with 2 mL of methanol/water (90/10, v/v). The eluate was dried in a centrifugal evaporator, redissolved in 250 μL assay buffer, and 2 × 100 μL were taken for analysis.

The four described methods were also performed with 1 mL plasma spiked with 4 fmol of ET standard and with 4 fmol ET standard in water. Extraction recovery for ET in plasma and in water was calculated by the amount of ET determined in radioimmunoassay (RIA). To control for different cartridge extraction recoveries of ET, as observed in the pilot experiments, we spiked one basal plasma sample from each person with a known definite endothelin standard (4 fmol) and performed cartridge extraction as described (method D). A RIA with an ET-1–specific assay system was performed (Amersham [ET1-21], cross-reactivities: 0.4% with big-ET, 52% with ET-3, 144% with ET-2; coefficient of variation: 5.4% ± 0.7% for within-assay precision, 9.5% to 17.3% for between-assay precision; IC50 = 6.8 ± 0.3 fmol/tube, IC50 = 3.6 ± 0.3 fmol/tube, IC50 = 1.6 ± 0.3 fmol/tube; 100 μL of endothelin standards or 100 μL unknown sample were mixed with 100 μL antiserum (rabbit anti-endothelin serum in assay buffer) in labeled polypropylene tubes and incubated for 4 h at 4°C. Then, 100 μL of tracer ([125I] ET-3, synthetic, Amersham, in assay buffer) were added, mixed, and incubated for 18 h at 4°C, and 250 μL of second antibody reagent (donkey anti-rabbit serum, Amersham) was added and incubated at room temperature for 10 min. For separating the bound fraction of the antibody centrifugation at 4°C for 10 min at 1500 × g was performed. Tubes were decanted and radioactivity was detected in a γ scintillation counter.

Statistics  Immunoreactive ET levels of normotensive and hypertensive subjects were compared with unpaired t test. A paired t test was used to compare ET levels before and after infusion and to compare ET levels in arterial and venous plasma. Results are given as mean ± SEM.

RESULTS

Twenty-four–hour ambulatory BP was significantly higher (P < .002) in patients than in healthy volunteers (systolic: 137 ± 2 vs 125 ± 2 mm Hg; diastolic: 84 ± 2 vs 75 ± 2 mm Hg). Other characteristics did not differ significantly (see Table 1). Arterial compliance as an indicator of vascular adaptive processes to increased luminal pressure was also similar in both groups; for details, see Reference 20.

The diameter of the radial artery was similar in hypertensive and normotensive subjects (2.9 ± 0.24 vs 3.09 ± 0.38 mm, NS) at baseline. The maximum increase in diameter during infusion of acetylcholine did not differ for hypertensives (3.4% ± 1.3%) and normotensives (2.3% ± 0.6%).

The different extraction recoveries for the methods tested in the pilot experiments are shown in Table 2. We found no significant difference in cartridge extraction recovery (method D) of endothelin between normotensives (61% ± 4% in arterial plasma, 71% ± 4% in venous plasma) and hypertensives (57% ± 3% in arterial plasma, 57% ± 5% in venous plasma).

Basal ET plasma levels overlapped between both groups, but were significantly higher in hypertensive (Figure 1) subjects (P < .01) than normotensive control subjects. The same was found when corrected for recovery (P < .01) (Figure 2).

Vasodilation induced by acetylcholine had no significant effect on ET plasma levels in hypertensive and normotensive subjects (Figure 3). There were no significant differences between arterial and venous ET
plasma levels at baseline and after vasodilation induced by acetylcholine any time of the experiment (Figure 3).

**DISCUSSION**

To test the hypothesis that ET formation is increased in young hypertensive patients compared with control subjects, we measured forearm arterial and venous ET levels. Endothelin concentrations were clearly higher in hypertensive patients than in control subjects, both in venous and arterial blood. Arterial and venous ET concentrations were not different from each other, and were not affected by local vasodilation.

The role of ET-1 in the development of high blood pressure is still far from being understood. In some forms of animal hypertension, mainly salt-sensitive forms like the DOCA-salt hypertensive rat, the Dahl salt-sensitive rat, and the ATII-infused rat, ET-1 is increased systemically. These forms are all characterized by severe hypertrophy of the small arteries. In humans, local production of endothelin-1 contributes to the maintenance of vascular tone, as the intravenous administration of an endothelin receptor antagonist increases forearm blood flow and lowers blood pressure. A genetic role for ET-1 in human hypertension is proposed by the fact that normotensive offspring of hypertensive parents exhibit enhanced plasma endothelin responses to mental stress. The recent finding that treatment with the endothelin receptor antagonist bosentan reduces blood pressure in mild-to-moderate hypertensives suggests a role for the peptide early in the development of human hypertension.

The possible role of ET-1 in human hypertension has been investigated by measuring the concentration of the peptide in plasma, and the results have been controversial. Some authors reported increased ET-1 concentration in essential hypertensive patients, whereas others found no difference between hypertensive and normotensive subjects. These controversial results have been attributed to increased ET-1 production in the presence of vascular injury and hypertensive target organ damage. However, our data do not support the hypothesis that end-organ disease accounts for the higher ET-1 plasma concentrations in hypertensive patients.

### Table 2. Extraction Recovery of 4 FMOL ET Standard in 1 ML Plasma or Water for Different Cartridges, Given as Mean ± SD (ND = NOT DETERMINED)

<table>
<thead>
<tr>
<th>Cartridge</th>
<th>Plasma Acidified</th>
<th>ET Standard in Water</th>
<th>ET Standard in Plasma</th>
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<tbody>
<tr>
<td>Amprep*C2</td>
<td>+</td>
<td>77 ± 45</td>
<td>47 ± 23</td>
</tr>
<tr>
<td>Bond-Elut*</td>
<td>+</td>
<td>ND</td>
<td>26 ± 12</td>
</tr>
<tr>
<td>SepPak*Vac/3ccC18</td>
<td>+</td>
<td>75 ± 25</td>
<td>10–43% (range, n &lt; 4)</td>
</tr>
<tr>
<td>SepPak*Vac/3ccC18</td>
<td>+</td>
<td>78 ± 18</td>
<td>61 ± 14</td>
</tr>
</tbody>
</table>

*ET = endothelin.*
istration in hypertensive patients. We studied young, untreated white men with no signs of target organ disease. These subjects, who were identified as hypertensive based on repeated casual blood pressure measurements, showed only mild hypertension during ambulatory blood pressure measurements. Our results and a previous report support the notion that plasma ET is increased early in the development of hypertension, and is not due to end-organ disease in these patients.

We believe that the differences in the methods used to measure plasma ET-1, in particular the different ET antibodies, are the most likely explanation for the controversial reports on plasma ET-1 concentrations in essential hypertension.12,13 (For more detail on the methodologic background, please see Technical Note).1

Physiologically, endothelin-1 is produced mainly in endothelial cells and its production is modulated by many factors, including mechanical ones such as shear stress and humoral ones such as angiotensin II, transforming growth factor, and many others. A precursor peptide is translated from the endothelin gene, which is cleaved by a furin-like enzyme to big-ET, and big-ET is subsequently converted to ET-1 by the endothelin-converting enzymes. The physiologic action of endothelin-1 is mediated by ET _a_ and ET _b_ receptors. The ET _a_ receptor is located on smooth muscle cells and its activation leads to vasoconstriction. ET _b_ receptors are located on endothelial cells and are known to stimulate the production of nitric oxide, prostaglandin, and adrenomedullin.26 In addition, ET _b_ receptors have been identified on smooth muscle cells, contributing to the vasoconstrictive effect of ET-1.27 Circulating ET-1 is cleared by the kidney, by enzymatic degradation, and by binding to the ET _a_ receptor.28,29

What could be the cause for elevated plasma immunoactive ET in early essential hypertension? ET-1 production could be increased on a transcriptional or translational cellular level; perhaps conversion of big-ET to ET-1 by the endothelin-converting enzymes ECE _a_ and ECE _b_ could be increased. However, in contrast to moderate or severe hypertensive subjects, endothelial preproET-1 mRNA expression is not increased in endothelium of small arteries obtained from gluteal subcutaneous biopsies of mild hypertensive subjects.30

Downregulation of ET _a_ receptors in resistance vessels and thus a reduced clearance might also be responsible for the observed increased plasma levels of endothelin-1 in early hypertensive subjects. This would be in agreement with the earlier described reduction in sensitivity to endothelin-1 of human subcutaneous resistance arteries of early hypertensive subjects in vitro,31 as the ET _a_ receptor contributes to vasoconstriction. Intraarterial administration of a bolus of endothelin-1 is known to produce transient ET _a_ receptor–mediated vasodilation.27 Thus, reduced initial vasodilation to boluses of endothelin-1 should be expected in early hypertension. As Kaufmann et al have shown, endothelin is also secreted by the pituitary in response to tilt.30 Although our patients had been supine for 30 min before the measurements, it is possible that the pituitary might have played a role in the different immunoreactive endothelin levels of essential hypertensive and normotensive subjects.

Measurements of plasma levels of immunoreactive ET will only provide limited insight into the role of a peptide that is a paracrine tissue hormone rather than an endocrine circulating hormone.1,2 It has been shown that endothelial cells secrete much more ET-1 towards the adjacent vascular smooth muscle cells than they do luminally.32 Unfortunately, our attempt to assess local vascular ET production from arteriovenous ET differences was not met with success, al-

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1Technical Note: Big-ET-1, the precursor of ET-1, is severalfold more abundant in human plasma than authentic ET-1.4–12 Antibodies raised against the N-terminal portion of ET-1 show significant cross-reactivity with big-ET-1, and possibly also ET-2.13 Immunoreactive ET-1 as measured by those N-terminal antibodies will mainly represent the precursor peptides rather than authentic ET-1.13 Most reports of unaltered plasma ET-1 in hypertensive subjects were based on ET-1 assays using N-terminal antibodies with high (>10%) cross-reactivity with big-ET-1.9–11 In these studies,9–11 the cross-reactivity with precursor peptides could thus obscure a higher ET-1 level in hypertensive subjects.

Endothelin-1 assays that avoid cross-reaction with big-ET precursors are better suited to detect differences in authentic ET-1.12,13 Theoretically, column extraction followed by HPLC and radioimmunoassay of specific fractions, which has become the standard method for peptides such as angiotensin II,33,35,36 might be considered the best method. Poor and variable recovery of ET peptides from HPLC columns12,13 has to date hampered this approach. We avoided the confounding problem of big-ET precursor peptides by using a C-terminal ET-1 antibody that shows only negligible cross-reactivity with big-ET.16 Using this approach, we and Shichiri et al demonstrated increased plasma ET-1 levels in hypertensive patients. A disadvantage of these antibodies is the high cross-reactivity with ET-2 and ET-3. However, Matsumoto et al have shown that ET-2 and ET-3 concentrations in human plasma are only a small fraction of ET-1 concentration (approximately 20% for ET-2 and ET-3 combined).

Including our own data, 16,27 but one of the studies that avoided the confounding factor of big-ET cross-reactivity have demonstrated increased ET plasma concentrations in essential hypertension. Using a sandwich assay that detects only ET-1 and ET-2, Miyauchi et al found higher ET-1 with male gender and increasing age but not with essential hypertension. Both Lemne et al and we studied only male subjects, and there were no age differences between normotensive and hypertensive individuals. Unfortunately, the short report of Miyauchi et al does not provide details on study subjects, collection and handling of samples, extraction procedures, and recovery from extractions, which might all affect the results. We found that some common procedures for column extraction, including the procedure recommended by the vendor of the assay we used, resulted in poor and highly variable recovery rates. We adapted the extraction procedure described by Löffler et al and performed a separate recovery for each sample, using unlabeled ET-1 standard. Reproducible recovery rates were thus obtained, and the comparison between hypertensive and normotensive subjects was not affected by the recovery.

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though ET-1 is produced locally in forearm vessels. Several factors may account for the lack of an arteriovenous difference of ET concentrations. First, ET-1 release in the circulation may represent only a spill-over of local production; second, simultaneous uptake and metabolism of ET-1 may obscure local release; finally, endogenous ET-1 production may occur too slowly to be detected during our sampling periods. The problems encountered here may resemble those of demonstrating local angiotensin production in blood vessels. There are no obvious arteriovenous differences but simultaneous correction for peptide uptake and degradation is necessary to uncover substantial local angiotensin production. Future research is necessary to elucidate this possibility.

In summary, our results show that an increased plasma ET concentration can be measured in young, untreated white essential hypertensive patients, as long as care is taken to standardize sample collection and handling, to ensure reproducible recovery of ET from plasma extraction, and to minimize cross-reactivity with big-ET precursor peptides. We studied that have avoided the confounding factor of plasma extraction, and to minimize cross-reactivity and handling, to ensure reproducible recovery of ET long as care is taken to standardize sample collection and metabolism of ET-1 may obscure local release.

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