Measurement of Plasma Endothelin-1 in Experimental Hypertension and in Healthy Subjects

Saad Abdel-Sayed, Juerg Nussberger, Jean-François Aubert, Peter Gohlke, Hans Rudolf Brunner, and Noureddine Brakch

**Background:** Endothelin-1 is an endothelium-derived potent vasoconstrictor peptide of 21 amino acids. To establish reference values in different models of hypertension and in human subjects an assay for plasma immunoreactive endothelin-1 (ET-1) was optimized.

**Methods:** ET-1 is extracted by acetone from 1 mL of plasma and subjected to a sensitive enzyme-linked immunosorbent assay.

**Results:** The detection limit for plasma ET-1 is 0.05 fmol/mL. Mean recoveries of the 1, 2, 5, and 10 fmol of ET-1 added to 1 mL of plasma were 66%, 75%, 85%, and 92%, respectively. Within-assay and between-assay coefficients of variation were ≤12% and ≤10%, respectively. Assay accuracy was demonstrated by consistent recoveries of added ET-1 over the entire physiologic range of plasma concentrations and by the linearity of ET-1 concentrations measured in serially diluted plasma extracts (r = 0.99). No ET-1 was detected when albumin buffer was extracted instead of plasma. Using this method, we found increased ET-1 levels in plasma of three experimental rat models of hypertension: stroke prone spontaneously hypertensive rats (SP-SHR), deoxycorticosterone acetate-salt hypertensive rats, and one kidney-one clip hypertensive rats. In contrast, plasma ET-1 levels of SHR were half those of normotensive Wistar rats. In two kidney-one clip hypertensive rats, plasma ET-1 concentrations were not different from those found in sham-operated control rats. Plasma ET-1 concentrations of 37 healthy men were 0.85 ± 0.26 fmol/ml (mean ± SD).

**Conclusions:** The present assay reliably measures ET-1 levels in rat and human plasma. It allows to discriminate between different forms of hypertension with high or low circulating levels of ET-1. Am J Hypertens 2003;16:515–521 © 2003 American Journal of Hypertension, Ltd.

**Key Words:** Endothelin, renovascular hypertension, DOCA-salt, enzyme-linked immunoassay.

The endothelins are a family of 21-amino-acid peptides with vasoactive, inotropic, and mitogenic properties. At present, three isopeptides, endothelin-1, endothelin-2, and endothelin-3, each with distinct genes and distributions in tissues, have been identified. Endothelin-1 appears to be the predominant isoform produced by the vascular endothelium; it acts mainly in a paracrine fashion on vascular smooth muscle cells where it causes long-lasting vasoconstriction.

Recent research has accumulated evidence for the clinical implications of endothelin. Plasma immunoreactive endothelin-1 (ET-1) concentrations have been shown to be of prognostic value in heart failure and acute myocardial infarction. Moreover, ET-1 has been suggested to be a marker for early reocclusion after percutaneous transluminal coronary angioplasty, for coronary arteriosclerosis and coronary endothelial dysfunction, for liver damage, and declining renal function. High plasma levels of ET-1 have been reported in different states of ischemia, hemodialysis, and essential hypertension. Elevated ET-1 has also been demonstrated during cardiac, liver, renal, and bone marrow transplantation. A significant role of ET-1 in the pathogenesis of cancer has recently been suggested.

Normal plasma concentrations of ET-1 are in the low picomolar range. Sensitive methods are needed for accurate measurement of this plasma peptide and for determination of differences in circulating levels of ET-1 in different physiologic and pathophysiologic states. The exclusion of cross-reacting material and related precursor or metabolite


From the Division of Hypertension and Vascular Medicine (SA-S, JN, J-FA, HRB, NB), University Hospital Lausanne Lausanne, Switzerland, and Institute of Pharmacology (PG), University Hospital of Schleswig-Holstein, Kiel, Germany.

This work was supported by the Cardiovascular Research Foundation Lausanne, Switzerland.

Address correspondence and reprint requests to Dr. Juerg Nussberger, Hypertension Division, CHUV, CH-1011 Lausanne, Switzerland; e-mail: juerg.nussberger@chuv.hospvd.ch
endothelin peptides present in plasma is required for specific estimation of ET-1. Reported plasma concentrations of ET-1 in healthy human subjects vary considerably with mean values ranging between 0.1 and 5.0 fmol/mL.3 Even a low detection limit of 0.2 fmol/mL may be insufficient without exclusion of cross-reactions.14 Li et al16 could not find increased circulating ET-1 levels in deoxycorticosterone acetate-salt hypertensive (DOCA-salt) rats using antibodies cross-reacting with endothelin-3 and big endothelin. Well-performing assays may be challenged because of high normal values if they use SepPak extraction without providing extraction blanks, and antisera which cross-react 52% with endothelin-3 and also with big endothelin.17

Careful sample handling to avoid in vitro artifacts and reliable extraction procedures to limit variation of recoveries are key elements required to obtain any meaningful quantitation. In the present study, we evaluated and modified a sensitive sandwich-enzyme immunoassay for plasma ET-1 based on commercially available materials. Preliminary experiments according to the manufacturer’s protocol had provided unsatisfactory recoveries decreasing from 88% to 30% when 2 and 10 fmol/mL of standard endothelin were added to plasma. Sample handling, extraction procedures, and buffer compositions had to be optimized to obtain reproducible results in the physiologic range. Using the modified new method we measured plasma ET-1 concentrations in normal control rats and in five different models of hypertensive rats (spontaneously hypertensive rats [SHR], stroke prone spontaneously hypertensive rats [SP-SHR], DOCA-salt, two kidney-one clip [2K1C], one kidney-one clip [1K1C]), as well as in healthy human subjects.

Methods

Blood Sampling

Five milliliters of blood were collected from the cubital vein of supine healthy male volunteers. Two milliliters of blood were collected from the femoral artery of conscious rats through an indwelling catheter. Blood was immediately transferred into prechilled glass tubes (ice bath) containing 0.054 mL of 15% tripotassium EDTA to achieve a final plasma concentration of 7 mmol/L. Blood was centrifuged at 4°C for 10 min at 1660 × g. Plasma aliquots of 1.2 mL were stored in polypropylene tubes at −80°C.

Extraction of ET-1 From Plasma

Endothelin is extracted from plasma as summarized in Fig. 1 (left panel). To 1 mL of plasma rapidly thawed under cold water stream we add 1.5 mL of acetone/0.2 mol/L HCl (87/13, v/v). After vigorous mixing for 1 min at room temperature, the mixture is kept at 4°C for 18 h and subsequently centrifuged at 1660 × g for 30 min at 4°C. The supernatant is stored under vacuum (Speed Vac, Savant, Basel, Switzerland) and the dry extract is redisolved in 0.5 mL of borate buffer (0.05 mol/L, pH 8.3) containing bovine serum albumin (Sigma, Buchs, Switzerland) at 5 g/L. The 200 μL extract or standard samples are pipet-

![FIG. 1. Left panel] Procedure of liquid phase extraction of endothelins from plasma before measurement of endothelin-1 by enzyme-linked immunosorbent assay (ELISA). [Right panel] Procedure of specific measurement of endothelin-1 by ELISA. BSA = bovine serum albumin.
Experimental Animals

Male Wistar rats and SHR of 8 weeks of age weighing 220 to 246 g were purchased from Iffa Credo (L’Arbresle, France) and SP-SHR were from the Institute of Pharmacology in Kiel (Germany). Rats were housed in transparent plastic cages in a quiet room at 22°C and maintained on standard rat chow containing 0.22% sodium and 0.20% potassium and tap water ad libitum. The Institutional Animal Care Committee approved the study protocols.

Twenty-four hours before blood sampling, rats were anesthetized by inhalation of 2% halothane in oxygen. Polyethylene (PE) catheters, consisting of PE-10 intravascular segments welded to PE-50 tubing were inserted into right femoral arteries for arterial blood pressure measurements and blood sampling. The PE-50 tubing were threaded under the skin and exteriorized at the back of the neck. The rats were housed individually in plastic cages and kept in a quiet place at 22°C.

On the study day, conscious rats were placed in a plastic tube for partial immobilization. Mean arterial pressure and heart rate were monitored after 20 min of rest, using the Notocord computerized system (Paris, France). Then 2 mL of arterial blood was collected and plasma was stored as described.

2K1C Hypertension Male 6-week-old Wistar rats weighing 160 to 170 g (n = 15) were anesthetized using 2% halothane in oxygen. An incision was made in the left abdomen, the left renal artery was isolated, and a Plexiglas clip (0.20 mm gap) was placed around the left renal artery (2K1C, n = 7). In control rats, the clip was subsequently removed (sham-operated, n = 8). After a 3-week observation period, mean arterial blood pressure was measured and blood samples were collected.

1K1C Hypertension Male 6-week-old Wistar rats weighing 170 to 180 g were used. To induce 1K1C hypertension (n = 9), a right nephrectomy was performed. The left renal artery was clipped as described previously. Control rats were prepared by performing a unilateral nephrectomy (sham-operated, n = 10). After a 3-week observation period, mean arterial blood pressure was measured and blood samples were collected.

DOCA-Salt Hypertension Male 6-week-old Wistar rats weighing 170 to 180 g (n = 9) were uninephrectomized under 2% halothane in oxygen anesthesia. A silicone tube (Cole-Parmer Instrument Co., Vernon Hills, IL) was prepared according to Wang et al.18 (28 mm length, 4 mm external diameter, 2.3 mm internal diameter; with 10 to 14 micropores of nearly 300 μm diameter on the wall). Each tube was filled with 80 mg of DOCA powder (Sigma Chemical Co., St. Louis, MO) and a silicone tubing of 2 mm in length was inserted into each end of the DOCA tube to keep the powder confined to the tube. This stopper tubing had an external diameter of 2.3 mm and an internal
diameter of 0.8 mm. One DOCA tube was implanted subcutaneously to each rat. They received 1% saline to drink. Rats became hypertensive and were studied 6 weeks after implanting the tubes. Uninephrectomized rats receiving tap water served as controls (n/H11005 9).

Statistical Analysis

Results are presented as means ± SD unless stated otherwise. Regression analysis was calculated by the method of least squares. Correlation coefficients for linear regression are presented. The unpaired Student t test was used to compare hypertensive rats with the controls. The significance level was P < .05.

Table 1. Within-assay coefficient of variation for increasing plasma ET-1 concentrations

<table>
<thead>
<tr>
<th>ET-1 (fmol/mL)*</th>
<th>n</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.84 ± 0.10</td>
<td>7</td>
<td>11.9</td>
</tr>
<tr>
<td>1.49 ± 0.07</td>
<td>5</td>
<td>4.7</td>
</tr>
<tr>
<td>2.33 ± 0.08</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>5.17 ± 0.13</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>9.94 ± 0.05</td>
<td>5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

ET-1 = immunoreactive endothelin-1; CV = coefficient of variation.
* Mean ± SD.

Table 2. Between-assay coefficient of variation for two human plasmas

<table>
<thead>
<tr>
<th>ET-1 (fmol/mL)*</th>
<th>n</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.02 ± 0.08</td>
<td>8</td>
<td>7.8</td>
</tr>
<tr>
<td>1.22 ± 0.12</td>
<td>7</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.
* Mean ± SD.

Results

Characteristics of Endothelin Assay

Fig. 2 shows the recoveries for acetone extraction of ET-1 from 1 mL of human plasma (mean ± SD, n = 5). Unspiked plasma contained 0.84 ± 0.10 fmol/mL ET-1 (n = 7). The mean recoveries were 66%, 76%, 86%, and 92% for added ET-1 at 1, 2, 5, and 10 fmol/mL, respectively. With increasing ET-1 content of the plasma, the precision of the measured ET-1 concentration increased.

Table 1 presents the within-assay coefficients of variation for multiple ET-1 measurements of five different plasmas with ET-1 concentrations ranging from <1 to 10 fmol/mL.
Table 3. Plasma immunoreactive endothelin-1 in different rat models of hypertension (mean ± SD)

<table>
<thead>
<tr>
<th>Rat Type</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Mean Arterial Pressure (mm/Hg)</th>
<th>Immunoreactive Endothelin-1 (fmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arithmetic Mean</td>
</tr>
<tr>
<td>Two kidney rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>9</td>
<td>225 ± 10</td>
<td>154 ± 8†</td>
<td>0.64 ± 0.08†</td>
</tr>
<tr>
<td>Wistar</td>
<td>9</td>
<td>230 ± 10</td>
<td>117 ± 9</td>
<td>1.20 ± 0.78</td>
</tr>
<tr>
<td>SP-SHR</td>
<td>10</td>
<td>240 ± 10</td>
<td>177 ± 6*</td>
<td>1.64 ± 0.13*</td>
</tr>
<tr>
<td>Wistar</td>
<td>10</td>
<td>246 ± 11</td>
<td>119 ± 13</td>
<td>1.17 ± 0.35</td>
</tr>
<tr>
<td>2K1C</td>
<td>7</td>
<td>289 ± 24</td>
<td>187 ± 11§</td>
<td>1.17 ± 0.28</td>
</tr>
<tr>
<td>Sham</td>
<td>8</td>
<td>331 ± 15</td>
<td>114 ± 7</td>
<td>1.33 ± 0.30</td>
</tr>
<tr>
<td>One kidney rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1K1C</td>
<td>9</td>
<td>291 ± 40</td>
<td>181 ± 22§</td>
<td>1.38 ± 0.22†</td>
</tr>
<tr>
<td>Sham</td>
<td>9</td>
<td>309 ± 19</td>
<td>116 ± 9</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>DOCA-salt</td>
<td>8</td>
<td>251 ± 31</td>
<td>197 ± 19§</td>
<td>2.57 ± 1.02§</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>356 ± 13</td>
<td>120 ± 6</td>
<td>0.60 ± 0.38</td>
</tr>
</tbody>
</table>

SHR = spontaneously hypertensive rats; SP-SHR = stroke prone–spontaneously hypertensive rats; 2K1C = 2 Kidney-1 clip Goldblatt hypertensive rats; 1K1C = 1 Kidney-1 clip hypertensive rats; DOCA-Salt = deoxycorticosterone acetate/saline rats.

* P < .01; † P < .001 v Wistar rats; ‡ P < .01; § P < .001 v sham-operated rats.

fmol/mL. These CV decreased with increasing concentrations of ET-1 from 11.9% to 0.5%.

The between-assay coefficients of variation for two human plasma samples are depicted in Table 2. They are situated at 7.8% and 9.8%.

The detection limit was defined as the ET-1 content read on the standard slope at three standard deviations above the optical density found in the absence of ET-1. The detection limit of the assay for 1 mL of plasma was 0.05 fmol/mL.

Assay accuracy is demonstrated by the close linear relationship between ET-1 added to and recovered from plasma (y = 0.93x − 0.24, r = 0.99; Fig. 2) and by the linearity of ET-1 concentrations measured in two different serially diluted plasma extracts (Fig. 3) Taking the reaction with ET-1 as 1.00, the cross-reactions with big ET-1, with ET-2 and with ET-3 were 0.000006, 1.020, and 0.048, respectively. No ET-1-like material was detected when the albumin-containing borate buffer was extracted instead of plasma.

Plasma levels of ET-1 in normal human subjects were 0.85 ± 0.30 fmol/mL (mean ± SD, n = 37), ranging from 0.37 to 1.57 fmol/mL.

Rat Hypertension Models

Table 3 shows plasma ET-1 concentrations as well as weight and mean arterial pressure of normotensive Wistar rats and of five different rat models of hypertension. Normotensive Wistar rats had plasma ET-1 levels of 0.7 to 2.1 fmol/mL. In SHR, plasma ET-1 levels were lower (P < .01) and in SP-SHR, they were higher than in normotensive Wistar rats (P < .001). Fig. 4 depicts blood pressure and plasma ET-1 levels in surgically induced models of hypertension. In hypertensive 1K1C rats, mean plasma ET-1 was increased 32% compared to sham-operated uninephrectomized rats (P < .002). The equally hypertensive 2K1C rats had plasma ET-1 levels that were not different from their controls but tended to be decreased (−11%; P = not significant). The DOCA-salt hypertensive rats exhibited 3.6-fold higher circulating ET-1 than normotensive uninephrectomized controls (P < .001).

Discussion

Among the circulating endothelins, their precursors, and metabolites, the 21-amino-acid vasoconstrictor isopeptide endothelin-1 appears to play a predominant role. Its plasma concentrations in the low picomolar range require careful sample handling to avoid in vitro generation or metabolism of the peptide, as well as a reliable extraction technique and sensitive methods to quantitate. Unlike its original commercial protocol, the modified assay described here fulfills these requirements. It enables accurate measurement of human plasma levels and allows to demonstrate differences in circulating ET-1 between various rat models of hypertension.

From 1 milliliter of EDTA–plasma endothelins are extracted into cold acetone during an extended (18 h) period, whereas proteins and enzymes generating or metabolizing ET-1 are precipitated. The procedure is characterized by reasonably low CV and >50% recoveries of physiologic plasma ET-1 concentrations. For higher plasma ET-1 concentrations, mean recoveries are further increased and the CV become very small (Table 1).

The specificity of our ET-1 measurement is provided by the sandwich-type immunoassay. The capture antibody excludes big endothelins, and the detection antibody hardly recognizes endothelin-3 (5% cross-reaction), which only represents 10% of circulating endothelins; the fully cross-reacting endothelin-2 is virtually absent in plasma.19–22 The absence of any interference is also confirmed by linearity of measured ET-1 levels in serially diluted plasma extract (Fig. 3), because any cross-reacting material would disturb the
linearity derived from a pure endothelin-1 standard slope.19,21,22 Several findings demonstrate the accuracy of our ET-1 measurement. First, serial dilution of plasma extracts yield perfectly linear endothelin-1 concentrations and the regression slopes show practically no y-axis intercept (Fig. 3). This virtually excludes the presence in the assay of any interfering or cross-reacting material. Second, buffer extracts do not contain any ET-1 and thus, exclude blank interference due to extraction or washing procedures. Third, repeat measurements of ET-1 concentrations in different plasmas provide consistent results. Both within-assay and between-assay CV are consistently below 12% within the tested range.

The detection limit of the assay for plasma ET-1 is 0.05 fmol/mL. Normal human plasma levels of ET-1 are 0.4 to 1.6 fmol/mL. They are in agreement with low picomolar normal values published by other investigators, who sometimes corrected their results for recovery losses.14–17,23–27 Not surprisingly, among these established assays for ET-1, the highest normal values were measured by assays which also included some cross-reacting big endothelins or endothelin-3; in addition, nonspecific blank interference was never excluded.17,27 Using our assay method, we demonstrate increased ET-1 levels in the plasma of three experimental rat models of hypertension. Plasma ET-1 concentration was higher in SP-SHR than in normotensive Wistar rats, and DOCA-salt hypertensive rats exhibited four times higher ET-1 levels than sham-operated control rats. This contrasts with the findings of Li et al.16 They could not find increased circulating ET-1 levels in DOCA-salt rats using antibodies cross-reacting with endothelin-3 and big endothelin.16 Our 1K1C hypertensive rats showed a moderate increase in ET-1 levels compared to sham-operated controls (Table 2). In contrast, the ET-1 level in the plasma of SHR was only half the level of normotensive Wistar rats. Schiffrin et al25 did not find such a difference between Wistar and SHR, but they used the SepPak extraction method and antiserum cross-reacting with big endothelin-1 and endothelin-3 at 10% and 7%, respectively. In 2K1C Goldblatt hypertensive rats, a renin-dependent model of hypertension, no increase in plasma ET-1 was observed. These findings complement and extend those reported by Schiffrin25,29 and Sventek30 and their colleagues. These researchers demonstrated an increased content of ET-1 and of preproendothelin-1 mRNA in the vascular wall of SP-SHR, DOCA-salt, and 1K1C hypertensive rats, but not in 2K1C or SHR hypertensive rats. Thus, our modified extraction and quantitation procedures of plasma ET-1 made it possible to measure increased plasma ET-1 levels in some forms of hypertension (SP-SHR, DOCA-salt, 1K1C), but not in other forms (2K1C, SHR).

In conclusion, ET-1 concentration in 1 mL of plasma can be reliably measured by acetone extraction and subsequent ELISA. Normal plasma ET-1 levels in humans and rats are in the low picomolar range. Significant differences in plasma ET-1 levels are found in different models of experimental hypertension.

Acknowledgments
We thank Ms. Catherine Amstutz for excellent technical assistance.

References


