Direct enzyme immunometric measurement of plasma big endothelin-1 concentrations and correlation with indicators of left ventricular function

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Recent studies have suggested that the plasma concentrations of endothelin-1, a potent vasoconstrictive peptide, are increased in patients with congestive heart failure. This study aimed to evaluate a new direct ELISA for big endothelin-1 (the precursor of endothelin-1), in comparison with a big endothelin-1 ELISA using plasma sample extraction, and to investigate whether plasma big endothelin-1 concentrations correlate with indicators of left ventricular function. The direct ELISA yielded significantly (P < 0.001) lower results than the assay with extracted samples (0.9 ± 0.5 pmol/L vs 2.7 ± 1.9 pmol/L; n = 90); however, the results of the two assays were closely correlated (r = 0.86, P < 0.001). Plasma big endothelin-1 concentrations exhibited a significant (P < 0.001) negative correlation (r = −0.46, r = −0.40) with the left ventricular ejection fraction and a significant positive correlation (r = 0.36, P < 0.01; r = 0.36, P < 0.01; r = 0.42, r = 0.38, P < 0.001) with the left ventricular end-diastolic pressure and the left ventricular end-diastolic (r = 0.42, r = 0.38, P < 0.001) and end-systolic (r = 0.52, r = 0.47, P < 0.001) volume indices. Plasma big endothelin-1 concentrations were notably greater in patients with New York Heart Association (NYHA) class II–IV symptoms than in patients without cardiac disease or in patients categorized to NYHA class I. These data suggest that plasma big endothelin-1 concentrations, measured by a direct ELISA, correlate with hemodynamic indicators and symptoms of left ventricular dysfunction.

Patients and Methods

Patients
Ninety patients (61 men and 29 women; mean age, 61.4 years; range, 37–80 years) undergoing cardiac catheterization were investigated. Blood samples were drawn from the femoral artery at the beginning of cardiac catheterization after informed consent was obtained. The study was approved by the local ethics committee. Substantial coronary atherosclerosis, documented by coronary angiography, was found in 76 patients (1 vessel, n = 28; 2 vessels, n = 22; and 3 vessels, n = 26); mitral valve regurgitation was present in three patients, and two patients had aortic valve stenosis. Patients were grouped according to the

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NYHA (New York Heart Association) classification [NYHA I: n = 55, mean age (± SD) 60.5 ± 10.0 years; NYHA II: n = 11, 64.4 ± 12.7 years; NYHA III: n = 12, 64.6 ± 10.3 years; NYHA IV: n = 3, 61.0 ± 16.1 years]. In nine patients (58.4 ± 10.8 years), a cardiac disease had been excluded. Some patients had a history of hypertension (n = 41) or hyperlipidemia (n = 39). Patients with renal disease, liver disease, and diabetes mellitus were excluded from the study. All patients had normal sinus rhythm at the time of blood sample collection. Medication, including angiotensin-converting enzyme inhibitors, diuretics (n = 5), aspirin (n = 57), β-adrenergic antagonists (n = 54), calcium channel blockers (n = 7), digitalis (n = 7), diuretics (n = 32), and nitrates (n = 40), was discontinued at least 12 h before the investigation.

CARDIAC CATHETERIZATION
Left-sided cardiac catheterization was performed with a 6 F pigtail catheter (Cordis), and a 7 F multipurpose catheter (Cordis) was used for right-sided cardiac catheterization. Left ventricular ejection fraction and left ventricular end-diastolic and end-systolic volumes were evaluated by left ventricular angiography. Cardiac output was determined by the Fick method. Pulmonary arterial pressure was measured with standard cardiac catheterization laboratory equipment (Metek).

MEASUREMENT OF PLASMA BIG ENDOTHELIN-1
Blood samples were collected in EDTA-containing tubes and immediately centrifuged; the plasma was stored at −80 °C. Plasma big endothelin-1 concentrations were measured with two different ELISAs: a direct assay without sample extraction, and an assay after solid-phase extraction. The direct assay (Biomedica) exhibited the following cross-reactivities as evaluated by serial dilutions of synthetic peptides and expressed as the mean relative immunoreactivity compared with big endothelin-1: big endothelin-1 (1–38) 100%, big endothelin-1 (22–38) <1%, endothelin-1 <1%, endothelin-2 <1%, and endothelin-3 <1%. According to the manufacturer, the intraassay CV (determined by repeated measurements of plasma samples) was 4.9% (1.2 ± 0.06 pmol/L, n = 11) and 3.9% (6.7 ± 0.26 pmol/L, n = 11), respectively; the interassay CV was 6.9% (1.6 ± 0.11 pmol/L, n = 12) and 6.1% (6.5 ± 0.39 pmol/L, n = 12), respectively; and the detection limit was 0.025 pmol/L. The assay was performed in microwell plates coated with an immunooaffinity-purified polyclonal rabbit anti-big endothelin antibody. Calibrator or sample (200 μL) and a monoclonal mouse anti-big endothelin antibody were added to the wells and incubated for 6 h at 37 °C. The wells were washed with washing buffer, and 200 μL of horseradish peroxidase-conjugated anti-mouse IgG antibody was added. After an incubation period of 1 h at 37 °C and another washing step, tetramethylbenzidine was added to the wells as a substrate; after 30 min, the reaction was stopped by the addition of 1 mol/L sulfuric acid. Absor-

bances were determined with a microtiter plate spectrophotometer at 450 nm against 620 nm as reference.

For plasma sample extraction, Sep-Pak C18 cartridges (Waters) were activated by methanol and then acetic acid (40 mL/L). Plasma samples (2 mL) were acidified with acetic acid (40 mL/L), centrifuged, and applied to the columns. The absorbed peptide was eluted with 2 mL of an aqueous solution containing 880 mL/L ethanol and 40 mL/L acetic acid, and the eluate was dried under a stream of nitrogen (analytical recovery, determined by addition of the cold peptide: 91.3% ± 7.5%, n = 15, mean ± SD).

The sample residues were dissolved in assay buffer (250 μL), and big endothelin-1 concentrations were measured by another commercially available ELISA (Johnson and Johnson). Cross-reactivity (the immunoreactivity relative to big endothelin-1, based on the concentration giving 50% B/Bmax data provided by the manufacturer) was big endothelin-1 100%, big endothelin-1 (22–38) <0.012%, endothelin-1 <0.028%, endothelin-2 <0.006%, endothelin-3 <0.006%; intraassay CV was 6.7% (17.8 ± 1.2 pmol/L, n = 14; established in the present study by repeated measurements of a pooled plasma sample) and 5.9% (41.0 ± 2.4 pmol/L, n = 18); interassay CV (evaluated by the manufacturer by repeated measurements of calibrator solutions) was 9.5% (48.0 ± 4.5 pmol/L, n = 22) and 9.0% (97.5 ± 8.7 pmol/L, n = 22); detection limit of the assay was 3 pmol/L, corresponding to 0.38 pmol/L in the original plasma sample (eightfold concentration of the samples during the extraction procedure). Calibrators and samples (100 μL) were incubated in microtiter wells precoated with anti-big endothelin-1 (22–38) rabbit IgG (18 h at 4 °C). After a washing step, horseradish peroxidase-labeled Fab’ fragment of anti-big endothelin-1 (1–21) rabbit IgG was added and incubated at 37 °C for 30 min. Tetramethylbenzidine was added as a substrate; after an incubation of 15 min, the reaction was stopped by addition of 1 mol/L sulfuric acid, and the resulting color was read at 450 nm in a microtiter plate spectrophotometer.

STATISTICAL ANALYSIS
Results are expressed as means ± SD. The correlation between plasma big endothelin-1 concentrations, measured by the two different assays, and hemodynamic variables was evaluated by linear regression analysis. Results from the two ELISA systems were compared by means of the two-sample t-test. Plasma big endothelin-1 concentrations in patients categorized according to the NYHA classification were evaluated by one-way analysis of variance, followed by the Newman–Keuls test.

Results
The direct big endothelin-1 ELISA yielded significantly (P <0.001) lower results than the ELISA with extracted samples (0.9 ± 0.5 pmol/L vs 2.7 ± 1.9 pmol/L, n = 90); however, the results obtained with the two different assay systems were closely correlated (r = 0.86; P <0.001) (Fig. 1).

Plasma big endothelin-1 concentrations exhibited a
significant negative correlation with the left ventricular ejection fraction, and a significant positive correlation with the left ventricular end-diastolic pressure, the left ventricular end-diastolic and end-systolic volume indices, and the mean and end-diastolic pulmonary arterial pressure. Data are shown in Table 1 and in Figs. 2 and 3. No significant correlation was observed with cardiac index or systolic, diastolic, or mean blood pressure values.

In accordance with the significant correlation between plasma big endothelin-1 concentrations and hemodynamic indicators of left ventricular function, a significant increase of plasma big endothelin concentrations was observed in patients with symptoms of moderate and severe heart failure (direct ELISA: patients without cardiac disease, 0.5 ± 0.1 pmol/L; NYHA I, 0.7 ± 0.2 pmol/L; NYHA II, 1.1 ± 0.5 pmol/L; NYHA III, 1.2 ± 0.6 pmol/L; and NYHA IV, 2.1 ± 0.7 pmol/L; ELISA after sample extraction: patients without cardiac disease, 1.8 ± 0.3 pmol/L; NYHA I, 2.2 ± 1.0 pmol/L; NYHA II, 3.2 ± 1.6 pmol/L; NYHA III, 4.1 ± 1.9 pmol/L; and NYHA IV, 6.0 ± 2.3 pmol/L); significances and data are shown in Fig. 4.

Table 1. Correlation between plasma big endothelin-1 concentrations and hemodynamic variables.a

<table>
<thead>
<tr>
<th>Variable</th>
<th>Direct ELISA</th>
<th>Extraction ELISA results</th>
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<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>LVEF</td>
<td>-0.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVEDP</td>
<td>0.40</td>
<td>&lt;0.001</td>
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<tr>
<td>LVEDVI</td>
<td>0.42</td>
<td>&lt;0.001</td>
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<tr>
<td>LVESVI</td>
<td>0.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAP mean</td>
<td>0.52</td>
<td>&lt;0.001</td>
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<tr>
<td>PAP diast.</td>
<td>0.53</td>
<td>&lt;0.001</td>
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a n = 42 for PAP determinations; n = 90 for all others.

LVEF, left ventricular ejection fraction; LVEDP, left ventricular end-diastolic pressure; LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; PAP mean, mean pulmonary arterial pressure; PAP diast., end-diastolic pulmonary arterial pressure.

Discussion

In the present study, plasma big endothelin-1 concentrations were measured by a new direct ELISA and by an ELISA performed after plasma sample extraction. The direct assay yielded significantly lower results than the assay with sample extraction. These differences might in part be explained by different sources of peptide calibrator and big endothelin-1 antibodies or by differences in the detection of big endothelin-1 bound to plasma proteins (a recently published study suggested a plasma...
protein binding of ~65% [17]). However, a close correlation between the results obtained with the two different assay systems was observed, suggesting that alterations in circulating plasma big endothelin-1 concentrations are reflected by both assays similarly.

Previous studies have demonstrated that plasma endothelin-1 concentrations are increased in patients with congestive heart failure, and a significant correlation between plasma endothelin-1 concentrations and indicators of left ventricular function, such as left ventricular ejection fraction, and with pulmonary arterial pressure values was observed [4,7,9]. This study adds further evidence that plasma concentrations of the precursor peptide big endothelin-1 are also correlated to indicators of left ventricular function and to pulmonary arterial pressure, as well as to the symptomatic status of left ventricular dysfunction.

Until now, the physiological relevance of circulating endothelin remains unclear, because it is believed to act primarily as a paracrine or endocrine factor, and several authors have suggested that increased plasma concentrations may represent spillover from a locally increased endothelin production. However, previous studies have suggested that endothelin might also act as a circulating hormone [3], and that increased circulating endothelin in patients with congestive heart failure may represent a part of the neurohumoral compensatory response and thus may contribute to the maintenance of the circulation, along with other neurohormones such as norepinephrine, angiotensin, and vasopressin [8,18]. In support of this view are the observations of increased circulating endothelin concentrations in response to acute hemodynamic stress induced by upright posture [8]. Most of the above-mentioned studies, demonstrating increased plasma endothelin concentrations in patients with congestive heart failure, have been performed with radioimmunoassays cross-reacting with big endothelin (10–38%) [4–9]. Wei et al. [9] have demonstrated, by gel filtration chromatography, that circulating plasma endothelin in patients with severe congestive heart failure consisted of ~62% big endothelin compared with 0% in healthy subjects. These data suggested that endothelin synthesis and release may be increased in congestive heart failure. Pacher et al. [14,16] and Kiowski et al. [15] found increased big endothelin-1 concentrations, determined by a radioimmunoassay after Sep-Pak C18 extraction, in patients with congestive heart failure. Pacher et al. [16] also have demonstrated that plasma big endothelin-1 concentrations are strongly related to survival in patients with...
chronic heart failure. Because the direct big endothelin-1 assay can easily be performed in a routine laboratory, it might become a tool in the diagnosis or management of left ventricular dysfunction and pulmonary hypertension.

References