Immunoluminometric Assay for Measurement of the C-Terminal Endothelin-1 Precursor Fragment in Human Plasma

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Background: Endothelin-1 (ET-1), a potent vasoconstrictor, is difficult to measure because of its instability and its binding to receptors and plasma proteins. We report a rapid, robust way to indirectly quantify ET-1 release by measuring the C-terminal ET-1 precursor fragment (CT-proET-1) without an extraction step.

Methods: In plasma samples from healthy individuals, patients with congestive heart failure (CHF), and patients with sepsis, we measured CT-proET-1 with a sandwich immunoluminometric assay that uses 2 polyclonal antibodies to amino acids 168–212 of pre-proET-1. We also correlated CT-proET-1 concentrations with bigET-1 measurements.

Results: The assay yielded results within 3 h and showed linear dilution with an analytical detection limit of 0.4 pmol/L and an interlaboratory CV <10% for values >10 pmol/L. Ex vivo CT-proET-1 was stable (<10% loss of immunoreactivity) in EDTA-, heparin-, and citrate-plasma for at least 4 h at room temperature, 6 h at 4 °C, and in EDTA-plasma for at least 6 months at −20 °C. CT-proET-1 values followed a gaussian distribution in healthy individuals (mean, 44.3 pmol/L; range, 10.5–77.4 pmol/L) without significant differences between males and females. The correlation coefficient for CT-proET-1 vs age was 0.25 (P <0.0001). CT-proET1 was significantly (P <0.0001) increased in patients with CHF (median, 104 pmol/L; range, 50.8–315 pmol/L) and patients with sepsis (median, 189 pmol/L; range, 34.6–855 pmol/L). The correlation between CT-proET1 and bigET-1 for 43 samples was 0.80 (P <0.0001).

Conclusions: CT-proET measurement is a rapid and easy method for indirectly assessing the release of ET-1 in critically ill patients.© 2006 American Association for Clinical Chemistry

The vasoconstrictor endothelin-1 (ET-1), a 21-amino acid peptide, is the most abundant member of the family of endothelins including ET-1, ET-2, and ET-3. ET-1 is derived mainly from vascular endothelial cells and acts in an autocrine and/or paracrine manner, mediating vasoconstriction predominately by binding to its ETA receptors on the underlying smooth muscle cells (1). ET-1 is considered to play an important pathophysiologic role in various diseases, including cardiovascular dysfunctions and sepsis (2–6), but reliable measurement of ET-1 release in the circulation is difficult because of its short plasma half-life (1–2 min) (7–9) and cleavage by neutral endopeptidases (10). Because of its low plasma concentrations and binding to plasma proteins (11), extraction and concentration of ET-1 may be necessary before measurement for accurate results (12). Furthermore, reliable ET-1 quantification is hampered by technical difficulties (12).

ET-1 is derived from a larger precursor peptide, pre-proET-1 (212 amino acids; Fig. 1). After signal sequence removal, further proteolytic processing leads to production of bigET-1 (38 amino acids). ET-1 is finally excised from bigET-1 by the action of endothelin-converting enzyme (ECE-1) (13). Measurement of bigET-1, which has slower clearance, has been proposed as an alternative approach for estimation of ET-1 release (14), but alterations in plasma bigET-1 may also not reflect changes in ET-1 synthesis (15).

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1 Nonstandard abbreviations: ET-1, endothelin-1; ECE-1, endothelin-converting enzyme; CT-proET-1, C-terminal endothelin-1 precursor fragment; CHF, congestive heart failure; NYHA, New York Heart Association; and CI, confidence interval.

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Sheep were immunized initially with 100 μmol/L hydroxysuccinimide ester to keyhole limpet hemocyanin. Its conjugated form and thereafter with 50 μmol/L peptides codes for CT-proET-1 are shown. Labeled antibody; controlled (HPLC with a C18 column and absorbance measured at 220 nm) by JPT Peptide Technologies GmbH. The peptides were PCT15 (sequence, CRSSEEHLRQTRSETMRNSVKSSFHDPKLGKPSREYVTHNRAHW, representing positions 169–212 of pre-proET-1), PCW14 (sequence, CSRERYVTHNRAHW, representing positions 200–212 of pre-proET-1 plus an N-terminal cysteine residue), and PSW44 (sequence, SSEEHLRQTRSETMRNSVKSSFHDPKLGKPSREYVTHNRAHW, representing positions 169–212 of pre-proET-1).

**Antibodies**

We recently identified several novel peptides derived in vivo from the ET-1 precursor (16). These proET-1 fragments are more stable than ET-1 and might not be subject to rapid turnover. A potential lack of tight feedback control makes them possible diagnostic targets for indirect assessment of released amounts of ET-1.

We describe a new sandwich assay for measurement without prior extraction of the C-terminal endothelin-1 precursor fragment (CT-proET-1; amino acids 168–212; Fig. 1) found in human plasma.

**Materials and Methods**

**Peptides**

Three peptides related to pre-proET-1 were chemically synthesized, purified (HPLC; >90% purity), and quality controlled (HPLC with a C18 column and absorbance measured at 220 nm) by JPT Peptide Technologies GmbH. The peptides were PCT15 (sequence, CRSSEEHLRQTRSET, representing positions 168–181 of pre-proET-1 plus an N-terminal cysteine residue), PCW14 (sequence, CSRERYVTHNRAHW, representing positions 200–212 of pre-proET-1 plus an N-terminal cysteine residue), and PSW44 (sequence, SSEEHLRQTRSETMRNSVKSSFHDPKLGKPSREYVTHNRAHW, representing positions 169–212 of pre-proET-1).

**Immuoassay**

A chemiluminescence sandwich immunoassay with coated tubes was set up as follows: Purified anti-PCW14 antibody (1 g/L) was labeled by incubation with a 1:2 molar ratio of MACN-Akridinium-NHS-Ester (1 g/L; InvVent GmbH) for 15 min at room temperature. The reaction was stopped by addition of 1/10 volume of 1 mol/L Tris, and labeled antibodies were separated from free label by size-exclusion chromatography on a Shodex Protein KW 803 HPLC column (Waters). Tracer was produced by diluting the labeled antibody into assay buffer (300 mmol/L potassium phosphate, 50 mmol/L NaCl, 10 mmol/L sodium EDTA, 1 g/L bovine serum albumin, 1 g/L nonspecific sheep IgG, 0.9 g/L sodium azide, pH 7.4). Polystyrene tubes (Greiner) were coated with anti-PCT15 antibody (per tube, 2 μg of antibody in 300 μL of 50 mmol/L Tris, 100 mmol/L NaCl, pH 7.8) overnight at room temperature. Tubes were then blocked with 10 mmol/L sodium phosphate (pH 6.5) containing 3% Karion FP (Merck), 3 g/L bovine serum albumin and lyophilized. Dilutions of peptide PSW44 in normal horse serum (Sigma) served as calibrators with concentrations of 500, 250, 100, 50, and 10 pmol/L. The immunoassay was performed by incubating 50 μL of samples/calibrators and 200 μL of tracer in coated tubes under agitation for 2 h at room temperature (18–27 °C). Tubes were washed 4 times with 1 mL of Brahms washing solution (Brahms AG), and bound chemiluminescence was measured for 1 s per tube with a LB952T luminometer (Berthold). As controls, horse sera containing 75 pmol/L (control I) and 200 pmol/L (control II) standard peptide were added at the beginning and end of each assay run. The assay was termed Brahms SEVACON LIA®.

**Patients and controls**

EDTA-plasma samples from healthy individuals were collected from the members of a local health club as described in detail elsewhere (17, 18). In addition, healthy individuals were recruited from among the employees of a local biotechnology center. Six participants underwent overnight fasting followed by a 1-L water load the next
morning, with subsequent measurements for the remainder of the working day. Written consent was obtained from all healthy volunteers. After peripheral venipuncture, all blood samples were centrifuged and frozen in aliquots at \(-20 °C\) within 1 h. Results from individuals with known cardiovascular diseases were excluded from the reference interval by definition. In total, measurements of plasma samples derived from 326 healthy individuals were used to establish reference values.

EDTA-plasma samples from patients with congestive heart failure (CHF), sepsis, severe sepsis, and septic shock [as defined by the American College of Chest Physicians/Society of Critical Care Medicine consensus conference (19)] were collected according to ethical guidelines and were stored at \(-20 °C\) until further use. We measured CT-proET-1 concentrations in 77 patients with CHF [New York Heart Association (NYHA) class 1, \(n = 2\); NYHA class 2, \(n = 6\); NYHA class 3, \(n = 26\); NYHA class 4, \(n = 43\)] and 116 patients with sepsis.

**Results**

**Technological characterization of CT-proET-1 assay**

**Measuring range and precision.** A typical calibration curve is shown in Fig. 2A. The lower limit of detection (limit of the blank), as determined with horse serum (mean relative light units of 10 determinations plus 2 SD), was 0.4 pmol/L.

We determined the intraassay imprecision (CV) by measuring 17 human EDTA-plasma samples with concentrations of 11.6–317 pmol/L in 10 parallel measurements. The intraassay CV was <5% for all 17 samples covering the range of the calibration curve. We determined the interlaboratory imprecision by measuring in duplicate 19 human EDTA-plasma samples with concentrations of 11.7–206 pmol/L. These data were generated over 3 days by 3 different operators for 12 assay runs using 3 different lots of tracer, calibrators, and controls and 2 different lots of reagents.

**Correlation between CT-proET-1 and bigET-1**

To correlate CT-proET-1 with bigET-1 values, we performed parallel measurements with EDTA-plasma samples from 29 patients with sepsis and 14 healthy control volunteers. CT-proET-1 was measured as described above. BigET-1 measurements were performed with a commercial ELISA (Biomedica), according to the manufacturer’s instructions. Briefly, the assay was performed by incubating 50 \(\mu\)L of samples/calibrators and 200 \(\mu\)L of conjugate (horseradish peroxidase–labeled monoclonal anti-bigET-1 antibody) for 4 h at room temperature in wells of microtiter strips coated with a polyclonal sheep anti-bigET-1 antibody. After washing the wells 5 times with 300 \(\mu\)L of 1× wash buffer, we added 200 \(\mu\)L of the substrate 3,3’,5,5’-tetramethylbenzidine base to each well. After an subsequent incubation step of 30 min at room temperature, the enzymatic reaction was stopped by addition of 50 \(\mu\)L of a sulfuric acid–containing solution provided with the assay reagents. The amount of bound antigen–antibody complexes was quantified by use of a microplate spectrophotometer (Bio-Tek Instruments), with measurement performed at 450 nm. The range of the calibration curve was 0.33–9.0 pmol/L.

**Statistical analysis**

We performed statistical analyses with Graph Pad Prism 4.0 and MedCalc Statistical software and tested distribution with the Kolmogorov–Smirnov test. We compared parametric data with an unpaired two-tailed \(t\)-test or one-way ANOVA and nonparametric data with the Mann–Whitney \(U\)-test or the Wilcoxon signed-rank test. Correlation was done by Spearman rank correlation. \(P\) values <0.05 were considered significant.
of coated tubes in 2 different laboratories with different luminometers. The interlaboratory CV was <10% for all measured samples with a CT-proET-1 concentration >10 pmol/L (Fig. 2B).

A high-dose hook effect was observed when CT-proET-1 concentrations >378 000 pmol/L were added to horse serum. However, this concentration is ~450-fold higher than the highest sample concentration measured in this study.

**Linearity and accuracy.** Assay linearity was assessed by dilution experiments. Linear dilutions (up to 1:32) were tested in 10 EDTA-plasma samples (Fig. 3A). Measured concentrations were multiplied by the dilution factor and compared with the values for the undiluted samples. None of the 10 samples showed a deviation during dilution >20% of the original value.

Pooling of 5 EDTA-plasma samples with low CT-proET-1 concentrations with 5 EDTA-plasma samples with high CT-proET-1 concentrations in 5 different combinations gave a mean measured concentration that was 98% of the expected concentration (range, 94%–104%).

**Interference studies**

We measured the interference of several biological substances by adding the potential interferents to 8 samples, according to protocol recommended by the Clinical and Laboratory Standards Institute (formerly NCCLS). The assay was not influenced by bilirubin up to 753 μmol/L, hemoglobin up to 5.8 g/L, protein (albumin) up to 60 g/L, or heparin up to 8400 IU/L. The deviation of CT-proET-1 values for samples with added interferents compared with the same samples without added interferents was always <20%.

**Use of serum and heparin-, citrate-, and EDTA-plasma and stability of analyte**

Matrices other than EDTA-plasma were tested with 10 matched samples obtained from healthy controls. Serum consistently gave values that were <35% of those obtained with EDTA-plasma, which we attributed to possible degradation by proteases. Therefore, the assay is not suitable for measurement of CT-proET-1 in serum samples. Heparin-plasma gave significantly higher median values (54.9 pmol/L) than did EDTA-plasma (47.7 pmol/L; \( P = 0.014 \)) or citrate-plasma (47.3 pmol/L; \( P = 0.004 \)).

We evaluated the stability of the native analyte at room temperature, 4 °C, and −20 °C in EDTA-, citrate-, and heparin-plasma samples drawn in parallel from 5 different individuals. At room temperature the analyte was stable (<10% loss of immunoreactivity) in all 3 matrices for 4 h. Thereafter, the mean concentrations decreased (Fig. 3B). Using a one-phase exponential decay equation, we calculated a half-life of 42 h for the analyte in EDTA-plasma at room temperature. The same EDTA-, citrate-, and heparin-plasma samples showed ex vivo stability of CT-proET-1 at 4 °C (<10% loss of immunoreactivity) for at least 6 h. Thereafter, the mean measured values decreased from 93%, 95%, and 91% of the original measured concentrations to 12%, 18%, and 14% after 21 days for EDTA-, citrate-, and heparin-plasma, respectively. In EDTA-plasma, the half-life for the analyte at 4 °C was 80 h. We tested the stability of the analyte at −20 °C in 20 EDTA-plasma samples (range, 23.2–47.8 pmol/L) without added peptide. After storage for 3 and 6 months, the measured CT-proET-1 values were 105% (range, 99%–117%) and 108% (range, 101%–115%) of the original values, respectively. We therefore recommend that samples be used directly within 4 h when stored at room temperature.

![Fig. 3. Accuracy and stability of the CT-proET-1 assay.](image-url)

(A) linear dilution (up to 1:32) of 6 representative of 10 tested samples. (B), stability of CT-proET-1 in EDTA-, citrate-, and heparin-plasma stored at room temperature. Shown are the mean (SD) measured CT-proET-1 concentrations relative to the original values in 5 different samples. Hours or days are given.
temperature or within 6 h when stored on ice, or that they be stored at ≤20 °C until use.

In 8 EDTA-plasma samples, freezing and thawing 3 times had no influence on the measured concentration [mean values, 103% (range, 81%–112%) of the original values]. After further thawing cycles, the mean measured values decreased to 90% (fourth thawing), 85% (fifth thawing), and 79% (sixth thawing) of the initial values. We therefore recommend a maximum of 2 freeze-thaw cycles.

REFERENCE INTERVAL
In the 326 healthy individuals (150 male, 176 female) CT-proET-1 values followed a gaussian distribution with a mean (SD) of 44.3 (10.6) pmol/L [95% confidence interval (CI) of the mean, 43.1–45.4 pmol/L] and a range of 10.5–77.4 pmol/L. The 99th percentile of the healthy population was 72.8 pmol/L, the 97.5th percentile was 66.6 pmol/L, the 2.5th percentile was 24.8 pmol/L, and the 1st percentile was 17.4 pmol/L, as calculated according to the Clinical and Laboratory Standards Institute protocol. Mean CT-proET-1 concentrations in males and females were not significantly different (45.4 and 43.2 pmol/L, respectively; Fig. 4A). Further stratification of the population by age is shown in Table 1. Post hoc analysis by Newman–Keuls multiple comparison revealed significant differences between the CT-proET-1 values of the 3 younger age groups (17–44 years) and the CT-proET-1 values of the 3 older age groups (45–75 years) with higher values in the older subgroup. CT-proET-1 concentrations were significantly correlated with age (r = 0.25; 95% CI, 0.14–0.35; P <0.0001).

Fig. 4. CT-proET-1 values in healthy individuals and patients.
(A), distribution of CT-proET-1 values in 150 healthy men and 176 healthy women. The difference in the mean values (horizontal lines) between the 2 groups was not significant (NS). (B), CT-proET-1 values for 3 representative of 6 healthy individuals over a 9-h period. Arrows indicate a water load (1 L within 5 min) and a standardized meal (~1200 kcal over 45 min). ●, 50-year-old female, body mass index = 23 kg/m²; ▲, 31-year-old male, body mass index = 31 kg/m²; ▼, 33-year-old male, body mass index = 21 kg/m². (C), distribution of CT-proET-1 values in 77 patients with CHF and 116 septic patients compared with 326 healthy individuals. The median values are indicated by horizontal lines. Differences between all 3 groups are significant. (D), correlation between CT-proET-1 and bigET-1 values. Data are shown for 29 patients with sepsis (●; Spearman r = 0.68; 95% CI, 0.41–0.84; P <0.0001) and 14 healthy controls (○; Spearman r = 0.28; 95% CI, −0.30 to 0.72; not significant). The overall correlation for both groups was r = 0.80 (95% CI, 0.66–0.89; P <0.0001).
To evaluate intraday variations in healthy individuals, we monitored 6 participants (3 male, 3 female) from 0800 in the morning to 1700 in the evening, taking 15 consecutive samples for CT-proET-1 measurement over this period. At the first blood sample, all participants had been without water or food for 14 h. The values for each participant remained very stable with means (SD) between 34.7 (4.5) pmol/L and 53.9 (3.4) pmol/L over the observation time and were not influenced by water (1 L) or food intake (standardized lunch of ~1200 kcal) at defined time points (Fig. 4B).

**CT-proET-1 in critically ill patients and correlation with bigET-1**

Compared with healthy individuals, values of CT-proET-1 were significantly (P <0.0001) increased in 77 CHF patients and 116 septic patients. Median (range) values were 104 (50.8–315) pmol/L for cardiac patients and 189 (34.6 to 855) pmol/L for sepsis patients. The distribution of the data is shown in Fig. 4C.

Correlation data for CT-proET-1 and bigET-1 were available for 29 patients with sepsis. The correlation was significant (Spearman r = 0.68; 95% CI, 0.41–0.84; P <0.0001; Fig. 4D). Also shown is the correlation between CT-proET-1 and bigET-1 for 14 samples from healthy controls. For this control group, correlation analysis revealed no significant correlation (Spearman r = 0.29; 95% CI, −0.30 to 0.72). On the other hand, the overall correlation for both groups together was highly significant (r = 0.80; 95% CI, 0.66–0.89; P <0.0001).

Median (range) bigET-1 concentrations in the 43 samples investigated in the correlation study were 1.61 (0.06–23.2) pmol/L compared with CT-proET-1 concentrations of 115 (28.0–703) pmol/L.

**Discussion**

This novel sandwich immunoassay for measurement of the C-terminal endothelin-1 precursor fragment CT-proET-1 allowed precise measurement of the analyte even in the lower end of the reference interval described in this study. In contrast to mature ET-1, CT-proET-1 is stable in all plasma matrices, at room temperature for at least 4 h and at 4 °C for at least 6 h. Plasma samples therefore do not need to be drawn into prechilled tubes and can be used within 4 h at room temperature or 6 h on ice, making the method suitable for routine use. Hence, this assay offers considerable advantages over direct measurement of ET-1. Alternatively, EDTA-plasma samples can be stored at least for 6 month at −20 °C. Although the choice of anticoagulant had no influence on the stability of the analyte after the sample was taken, the use of heparin as anticoagulant gave significantly higher CT-proET-1 values compared with EDTA and citrate. This observation is consistent with reports of higher albumin concentrations in heparin-plasma compared with EDTA-plasma (20), possibly because of an osmotic effect of anticoagulants such as EDTA and citrate (20, 21). To clarify the clinical relevance of this deviation, a larger number of samples should be investigated. Citrate-plasma can be used if the laboratory defines reference intervals for this type of matrix.

Because mature ET-1 is thought to bind to receptors close to the site of release rather than being a circulating hormone (1), ET-1 assay results based only on plasma concentrations cannot be used to draw conclusions on ET-1 biosynthesis. Most reported assays require complex preanalytical procedures, including peptide extraction (12, 22), and recovery strongly depends on the method used for extraction and/or concentration (23), leading to uncertain results. According to some manufacturers of commercially available assays, samples can be assayed with no prior purification. However, because of ET-1 binding to residual proteins in plasma, purification is mandatory if samples contain low ET-1 concentrations (12). Although ET-1 is unstable, it can adhere to the surfaces of routinely used test tubes, thus affecting efficiency of the test assays (23). Moreover, reliable ET-1 quantification is hampered by technical difficulties such as cross-reactivity of assay antibodies against other endothelins because of strong sequence homologies (12, 22, 24).

Indirect assessment of t ET-1 release by measurement of the CT-proET-1 fragment offers a simple and readily available alternative. No extraction of the peptide is necessary, and the lack of sequence homologies excludes cross-reactivity of the assay antibodies against other members of the endothelin family. Cross-reactivity against full length proET-1 is theoretically possible, but proET-1 immunoreactivity is undetectable in human plasma (16).
Estimates of ET-1 release based on bigET-1 measurements showed good correlation with parallel CT-proET-1 measurements, affirming the suitability of the CT-proET-1 method. An advantage of the CT-proET-1 assay described here is that the CT-proET-1 fragment might be not subject to rapid turnover (16), whereas bigET-1 is an intermediary product in the biogenesis of ET-1 and is further cleaved by the action of ECE-1 and chymase into the active vasoconstrictor peptides ET-1 and ET-1(1–31) respectively. The ~70-fold higher concentrations of CT-proET-1 compared with bigET-1 shown in this study support this reasoning.

Because bigET-1 has been reported to be present in human plasma in concentrations as low as those of ET-1 (22), an extraction step before measurement is essential for many assays (22, 25, 26). An exception is the commercial bigET-1 assay used in this study, which is applicable for direct measurement of the analyte in human plasma. Nevertheless, because bigET-1 concentrations are lower (particularly in healthy controls), the imprecision of the bigET-1 assay used is likely to be higher than that of the CT-proET-1 measurements. According to the manufacturer, the interassay CV is 9% at 2.3 pmol/L; however, the median bigET-1 concentration in healthy individuals as stated in the assay manual is 10-fold lower (0.2 pmol/L), indicating a higher interassay CV for measurements within the reference interval. In comparison, the CT-proET-1 assay described here allows precise measurement of the analyte with an interassay CV <10% for all investigated samples, including those from healthy controls.

In healthy individuals, CT-proET-1 followed a gaussian distribution with a mean (range) of 44.3 (10.5–77.4) pmol/L. There was no difference between males and females, in accordance with the finding that bigET-1 in healthy humans is independent of sex (25). Stratification of the healthy population by age revealed a significant trend to higher CT-proET-1 values in older individuals. It cannot be ruled out that values in the older age groups are higher because of unidentified cardiovascular diseases. For the definition of the reference interval in healthy individuals, we excluded individuals with known cardiovascular diseases (mostly hypertension). Compared with healthy individuals, CT-proET-1 values in these individuals (n = 50) were significantly (P <0.0001) increased with a mean (range) of 54.9 (30.9–83.4) pmol/L. In healthy volunteers, CT-proET-1 values were stable during the day and were not influenced by either food or water intake. Nighttime values were not studied.

The mean CT-proET-1 concentrations observed in this study were ~40- to 100-fold higher than the reported concentrations for mature ET-1 [0.44 pmol/L (27) and 1.2 pmol/L (28)] and bigET-1 [0.45 pmol/L (29) and 0.47 pmol/L (25)] in healthy individuals. These findings again support the notion that CT-proET-1 is more stable in the circulation and less affected by rapid turnover than are ET-1 and bigET-1.

Measurement of a nonfunctional, stable peptide derived from the cognate precursor of a bioactive, rapidly cleared peptide has been successfully applied to the A- and B-type natriuretic peptides (30–34), the vasodilatory peptide adrenomedullin (17, 35, 36), insulin (37), and vasopressin (18, 38). A measurable peptide derived from the ET-1 precursor can improve the diagnosis, monitoring, and prognosis of diseases such as CHF, sepsis, severe sepsis, or septic shock, which were present in our study population, in which ET-1 is increased (27, 28, 39–42). In addition, accurate measurement of the true release of ET-1 may facilitate monitoring of ET-1 receptor antagonist therapy in patients with pulmonary artery hypertension (43, 44).

In conclusion, CT-proET-1 measurements may be useful for indirect monitoring of the release of ET-1 in patients with cardiovascular diseases, systemic inflammation, or sepsis and might be helpful in directing therapeutic measures.

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References


