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Specific Sandwich-type Enzyme Immunoassays for Smooth Muscle Constricting Novel 31-Amino Acid Endothelins

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We established highly sensitive and specific sandwich-enzyme immunoassays (EIAs) for three newly discovered bioactive 31-amino acid endothelins [ETs(1-31)], which can detect as little as 0.16 pg/well of ET-1(1-31), 0.39 pg/well of ET-2(1-31), and 0.16 pg/well of ET-3(1-31). The EIAs showed no crossreactivity with 21-amino acid endothelins [ETs(1-21)] or big ETs at the usual assay concentrations below 1–5 ng/ml. In reversed-phase HPLC, immunoreactive ETs(1-31) in the granulocytes of normal human subjects eluted at the exact positions of authentic ETs(1-31), except for the presence of one additional unknown immunoreactive ET-1(1-31). The results also indicate that ETs(1-31) exist in the granulocytes at levels higher than or similar to those of ETs(1-21). This study is the first to establish EIAs for novel bioactive ETs(1-31). These assays can be utilized to assess the pathophysiological roles of ETs(1-31). © 1999 Academic Press

Endothelins (ETs) are 21-residue, potent, smooth muscle-constricting peptides originally isolated from conditioned medium of cultured porcine aortic endothelial cells (1). So far they have been detected in various tissues and cells (2–6), exerting their effects in a paracrine or autocrine manner, and also found as circulating hormones (7). ETs comprise three isopeptides, ET-1, ET-2, and ET-3, and are generated from big endothelins through cleavage of the Trp^{22}-Val^{23} bond by endothelin converting enzyme-1 and -2 (8). Recently, we found novel, smooth muscle-constricting 31-amino acid endothelins, ETs(1-31), that are generated from big endothelins through the specific cleavage of the Tyr^{31}-Gly^{32} bond by human chymase (9). In addition, it may transiently be generated by other chymotrypsin-type proteases, such as human cathepsin G in granulocytes and rat mast cell chymases (9, 10). ETs(1-31) exhibit equivalent or lower contractile potencies in comparison with the 21-amino acid endothelins, ETs(1-21), and the effects are dependent on species, vessel type, and vessel size (9, 11, 12). Although pharmacological studies of the effects of ETs(1-31) on vascular and tracheal smooth muscle have been undertaken *in vitro* using synthetic peptides (9, 11–14), there is no direct evidence concerning the presence of ETs(1-31) in human tissues, cells, and serum.

In order to study the physiologic and pathophysiologic roles of these newly-discovered bioactive ETs(1-31), specific and sensitive assay methods are imperative. In the present study, we established for the first time sensitive and specific sandwich EIAs for ET-1(1-31), ET-2(1-31), and ET-3(1-31), that do not cross react with ETs(1-21) and big ETs. Using these EIAs, we analyzed the intracellular levels of ETs(1-31) in human granulocytes.

MATERIALS AND METHODS

Materials. Human big ET-1, -2, and -3, and ET-1, -2, and -3(1-21) were purchased from the Peptide Institute (Osaka, Japan). ET-1, -2, and -3(1-31) were synthesized by solid-phase procedures by the Peptide Institute. EIA assay kits for ET-1(1-21), ET-3(1-21), big ET-1, and big ET-3, anti-human ET-1 Fab'-HRP conjugate, which equally recognizes the N-terminal loop domains of ET-1 and ET-2, and anti-human ET-3 Fab′-HRP conjugate were purchased from Immuno-Biological Labs (Fujioka, Japan). Bovine thyroglobulin and tetramethyl benzidine was from Sigma (St. Louis, MO, USA), and activated thiol Sepharose 4B was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, England). All other reagents were commercial products of the highest grade available.

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Abbreviations used: ET, endothelin; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; ACN, acetonitrile.

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Immunization. Immunogen peptides composed of C-terminal heptapeptides, ET-1, -2, and -3(25-31), and cysteine at the N-terminus to conjugate the hapten peptide to the carrier protein, were synthesized by the solid phase method with an automated peptide synthesizer (Model 430A, Applied Biosystem Co., USA). Maleimide groups were introduced into bovine thyroglobulin by reaction with N-(6-maleimidocaproyloxy) succinimide (Dojin, Kumamoto, Japan), after which the samples were dialyzed (15). Then, the maleimide activated thyroglobulin (10 nmol) was reacted with 3 μmol of the immunogen peptides at 4°C for 24 h. The conjugates obtained were emulsified with an equal volume of complete Freund's adjuvant for the first injection and with incomplete Freund's adjuvant for booster injections and injected into rabbits (100 μg/rabbit) 7 times at 2 week intervals. The animals were bled one week after the last booster immunization.

Sandwich-EIA for ETs(1-31). Rabbit anti-human ETs(1-31) antibodies were fractionated by affinity chromatography on a column of immunogen peptide-coupled thiol Sepharose 4B (2 mg of each peptide was coupled to 1 g of thiol Sepharose 4B). The monospecific antibody fraction was eluted with 0.2 M glycine-HCl, pH 2.5, and dialyzed against PBS. In sandwich-EIAs for ETs(1-31), monospecific C-terminal ETs(25-31) antibodies were used as immobilized antibodies. Microtiter plates were treated with antibodies against the C-terminal domain of each ET(1-31) at a concentration of 20 μg/ml (100 μl) in 0.1 M carbonate buffer, pH 9.6. The plates were left to stand at 4°C for 1 day and stored at 4°C with 300 μl/well of 1% BSA in PBS. In the EIA, authentic ETs(1-31), other related ET derivatives, or samples to be tested in 100 μl of buffer A (PBS containing 1% BSA and 0.05% Tween 20) were added to each well and incubated overnight at 4°C. The wells were washed 7 times with PBS containing 0.05% Tween 20, and then 100 μl of anti-human ET-1 Fab′-HRP conjugate, which reacts equally with the N-terminal loops of ET-1 and ET-2, or anti-human ET-3 Fab′-HRP conjugate, which specifically recognizes the N-terminal loop domain of ET-3, were added to each well at a final concentration of 1.9-3.3 μg/ml in buffer A. The plates were then incubated at 37°C for 30 min, washed 9 times with PBS containing 0.05% Tween 20, and the bound enzyme activity was measured using tetramethyl benzidine as a chromogen.

Preparation of human granulocytes. Since cell death or tissue excision leads ETs to be rapidly degraded intracellularly with concomitant formation of many unusual immunoreactive ET derivatives, the cells to be tested should be viable or tissues should be frozen immediately after excision. In this study, we analyzed the intracellular levels of ETs(1-31) as well as ETs(1-21) and big ETs in viable human granulocytes. Human granulocytes were isolated from the buffy coat of healthy donors using Lymphoprep from Nycomed (Oslo, Norway) according to the manufacturer's protocol. The purity of the isolated granulocyte fraction, determined by morphologic examination, was more than 95%, with more than 98% viability as determined by trypan blue dye exclusion. The collected granulocytes were immediately frozen and kept at −80°C until use.

Extraction of ET derivatives. Frozen granulocytes (3×10^8 cells) were suspended in 300 μl of ice cold water containing 2 mM leupeptin, 2 mM benzamidine, and 10 mM PMSF and were allowed to stand on ice for 5 min to complete the formation of the intracellular protease-protease inhibitor complex. Then the cells were lysed by sonication for 5 min in 30 ml of 0.6N HCl containing 0.1% TFA, immediately boiled for 10 min, and homogenized again with 10 strokes of a pestle in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 25,000 × g for 30 min at 4°C and the supernatants were collected. To complete the extraction of ETs with hydrophobic properties, the precipitates were extracted again by homogenizing with 20 ml of 80% ACN containing 0.1% TFA and then centrifuging at 25,000 × g for 30 min. The ACN concentration of the combined supernatants was brought to 20% by the addition of 0.1% TFA and the supernatant was applied to an Amprep 100 mg C2 column (Amersham Pharmacia Biotech) that had been prewashed sequentially with methanol, distilled water, and 0.1% TFA. The columns were then washed sequentially with 2 ml of 0.1% TFA and 2 ml of 20% ACN containing 0.1% TFA. The materials adsorbed were eluted with 2 ml of 80% of ACN containing 0.1% TFA and concentrated in siliconized plastic tubes to about 200 μl under reduced pressure using a SpeedVac concentrator taking care of insoluble ET derivatives upon complete drying. The recoveries of the extracted ET derivatives, analyzed by the addition of internal ^{125}I-ET derivatives, were 84%, 87%, 83%, 89%, 86%, 86%, 80%, 86%, and 86% for ^{125}I-ET-1(1-21), ^{125}I-ET-2(1-21), ^{125}I-ET-3(1-21), ^{125}I-ET-1(1-31), ^{125}I-ET-2(1-31), ^{125}I-ET-3(1-31), ^{125}I-big ET-1, ^{125}I-big ET-1, and ^{125}I-big ET-3, respectively, indicating that these ET derivatives could be recovered well from the column.

Separation of nine ET derivatives by reversed-phase HPLC. The cell extracts (300 μl) were diluted with the same volume of dimethyl sulfoxide containing 0.1% TFA, centrifuged, and loaded onto a TSK ODS-120T column (4.6 mm × 250 mm, Tosho, Tokyo, Japan). The elution solvents used were A, 0.1% TFA and B, 90% ACN containing 0.1% TFA. During elution, the concentration of B was increased linearly from 0 to 32% over 25 min, held at 32% for 165 min, and then raised from 32 to 100% over 20 min at a flow rate of 1.0 ml/min. Nine synthetic ET derivatives were well separated by isocratic elution with solvent of B at 32% (28.8% ACN in 0.1% TFA); 1-ml fractions were collected in siliconized tubes, combined with the eluate emerging at the elution time of each standard synthetic ETs, and concentrated to about 200 μl under reduced pressure taking care not to complete drying. BSA (500 μg) was added to each fraction and then the samples were lyophilized and assayed for immunoreactive ETs by sandwich-EIAs. To avoid contamination by ETs in the previous sample, the column was washed 3 times between use.

RESULTS

Specific Sandwich-EIAs for ETs(1-31)

Representative standard curves for ETs(1-31) and curves for crossreaction with corresponding ETs(1-21) and big ETs in sandwich-EIAs for ETs(1-31) are shown in Fig. 1. The EIAs for ET-1, -2, and -3(1-31) could detect as little as 0.16 pg/well for ET-1(1-31), 0.39 pg/well for ET-2(1-31), and 0.16 pg/well for ET-3(1-31). These EIAs for ETs(1-31) specifically detected each authentic ET(1-31) without crossreactivity (less than 0.1%) with ETs(1-21) or big ETs at the concentrations below 1-5 ng/ml. The EIAs for ET-1(1-31), ET-2(1-31), and ET-3(1-31), however, crossreacted slightly with big ET-1 (3.4 ± 0.1%), big ET-2 (3.4 ± 0.04%), and big ET-3 (2.58 ± 0.02%), respectively, at concentrations of 5 ng/ml, but not with ETs(1-21) as shown in TABLE 1. Although a sensitive sandwich-EIA for ET-1(1-21) consisting of two antibodies directed against the N-terminal and C-terminal portions of ET-1(1-21) has been reported, the EIA for ET-1(1-21) fully crossreacted with ET-2(1-21) because of the sequence identity of the C-terminal portions of ETs(1-21) (2, 16). In comparison with the EIAs for ETs(1-21), the EIAs for ETs(1-31) could specifically detect each ET(1-31) without crossreactivity because of specific antigenic epitopes in these C-terminal portions. These results indicate that the newly discovered ETs(1-31) are separately quantified by these high sensitive EIAs.

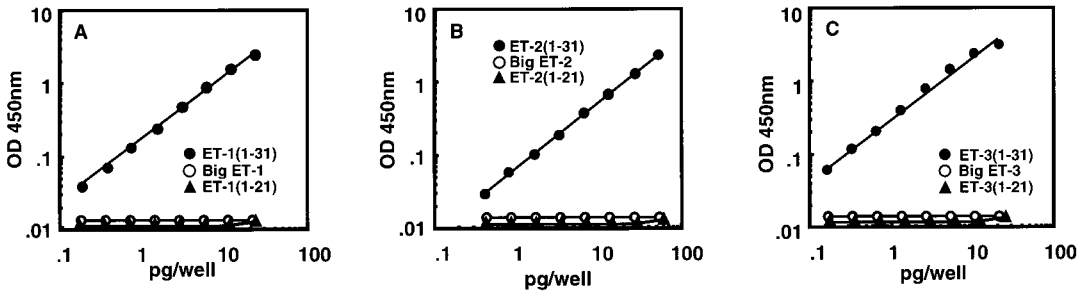

FIG. 1. Standard curves of synthetic human ETs(1-31) in sandwich-EIAs for (A) ET-1(1-31), (B) ET-2(1-31), and (C) ET-3(1-31) and the crossreactivities of EIAs for the corresponding ET(1-21) and big ET.

Characterization of Immunoreactive ETs(1-31) and Their Levels in the Granulocytes of Normal Human Subjects

Immunoreactive ETs(1-31), as well as other ET derivatives in the granulocytes of normal subjects, were characterized by reversed-phase HPLC on an ODS-120T column (Fig. 2). The recoveries of ^{125}I-labeled ET-1, -2, -3(1-21), ET-1, -2, -3(1-31), big ET-1, -2, and -3 were 83.5%, 75.4%, 78.5%, 71.3%, 71.7%, 71.5%, 72%, 72.4%, and 60.8%, respectively. Immunoreactive ET-2 and -3(1-31) emerged at the elution time of the corresponding authentic ETs(1-31), indicating that the immunoreactive ET-2 and -3(1-31) detected by the EIAs are identical to ET-2 and -3(1-31) themselves. Immunoreactive ET-1(1-31), however, exhibited two major peaks, one of which, observed at 143 min, corresponded to authentic ET-1(1-31) while the other peak at 95 min did not correspond to any reported ET derivatives.

TABLE 1

The Crossreactivities of EIAs for ET-1(1-31), ET-2(1-31), and ET-3(1-31)

	Crossreactivity (%)		
Antigen	ET-1(1-31) assay	ET-2(1-31) assay	ET-3(1-31) assay
ET-1(1-21)	≤0.1	≤0.1	≤0.1
ET-2(1-21)	≤0.1	≤0.1	≤0.1
ET-3(1-21)	≤0.1	≤0.1	≤0.1
ET-1(1-31)	100	≤0.1	≤0.1
ET-2(1-31)	≤0.1	100	≤0.1
ET-3(1-31)	≤0.1	≤0.1	100
Big ET-1	3.4 ± 0.1	≤0.1	≤0.1
Big ET-2	≤0.1	3.4 ± 0.04	≤0.1
Big ET-3	≤0.1	≤0.1	2.58 ± 0.02

Note. The cross reactivities of EIAs for ET-1(1-31), ET-2(1-31), and ET-3(1-31) were analyzed in the presence of various ET derivatives at concentrations of 5 ng/ml. Values (n = 5) are expressed as the percentage of the mean reactivity ±SE for each ET(1-31).

The immunoreactive ETs(1-21), big ET-1 and big ET-3 in the eluate from reversed-phase HPLC were also detected by commercially available sandwich-EIAs. The level of big ET-2 was not analyzed because EIA for big ET-2 is not available. As shown in Fig. 3, the levels of big ETs, ETs(1-21), and ETs(1-31) analyzed are in the range of about 35 to 110 pg/10^8 cells. The levels of ET-1 and -3(1-31) are higher than those of ET-1 and -3(1-21) and the level of ET-2(1-31) is in the similar range to that of ET-2(1-21).

DISCUSSION

The present study demonstrates highly sensitive and specific sandwich-EIAs for newly discovered bioactive peptides, ETs(1-31), which consist of two antibodies directed against the specific antigenic epitopes of the N- and C-terminal portions of each ET(1-31). The concentrations of ETs(1-21) and big ETs in plasma and tissues as analyzed by EIAs and radioimmunoassays have been reported (2, 16–24), but the data show considerably variations. We repeated several published procedures for the extraction and measurement of ET derivatives by EIA and radioimmunoassay in cells and tissues and found that extraction efficiency and insoluble residues containing ETs that appear during the process of measurement are the main reasons for the differences in the data. Another reason is compounds that interfere with the EIA in tissue extracts and plasma. Therefore, we developed an effective method for the measurement of cellular ETs(1-31), ETs(1-21), and big ETs by EIA.

ETs were extracted from human granulocytes under acidic conditions with at least 50 volumes of 0.6N HCl containing 0.1% TFA and again extracted from the residue of the first extraction with 80% ACN containing 0.1% TFA for complete extraction. This protocol achieves about 96% extraction from the cells as determined by the addition of internal ^{125}I-ET derivatives (data not shown). Compounds interfering with the EIA in the samples were reduced by sequential washing of

the C2 column with 0.1% TFA and 20% ACN containing 0.1% TFA. In the process of sample preparation for EIA, we avoided sample drying to minimize the formation of insoluble residues, and used siliconized plastic tubes to protect against the nonspecific adsorption of ETs. Since nine different ET derivatives did not separate well on a reversed-phase C_{18} column under the conditions reported (2, 22–24), we used isocratic elution with 28.8% ACN in 0.1% TFA for 165 min, and this protocol enabled us to achieve the complete separation of these ET derivatives.

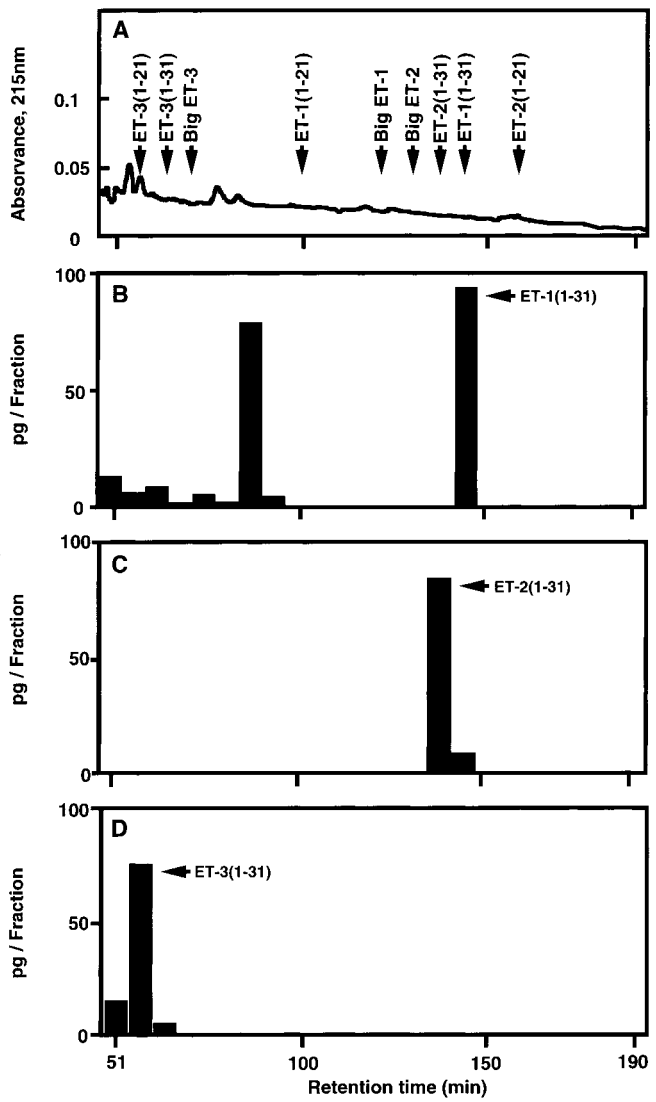

FIG. 2. Reversed-phase HPLC profiles of extracts of human granulocytes on a TSK ODS-120T column. Various ET derivatives were extracted from human granulocytes (3×10^8 cells) and separated by reversed-phase HPLC on an ODS-120T column as described under "Materials and Methods." Elution was monitored at 215 nm (A). Each fraction was subjected to sandwich-EIAs for ET-1(1-31) (B), ET-2(1-31) (C), and ET-3(1-31) (D). Arrows indicate the eluted positions of nine authentic, different ET derivatives.

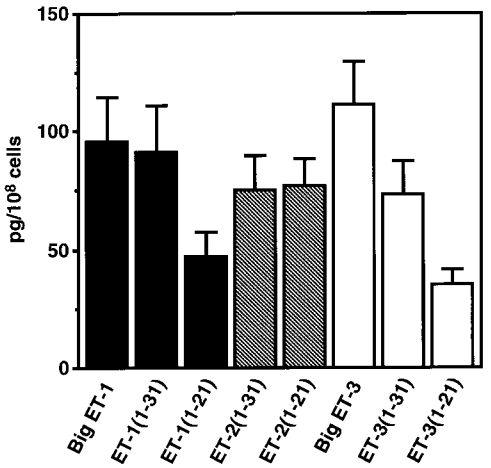

FIG. 3. Comparison of the levels of immunoreactive ET derivatives in human granulocytes. A human granulocyte extract was subjected to reversed-phase HPLC as shown in the legend to FIG. 2, and the amounts of ET derivatives in the fractions were measured by EIAs. The ET values were then corrected for the recovery efficiencies of the Amprep C2 and ODS-120T columns and expressed as the mean ± SE/10^8 cells (n = 10).

This study clearly demonstrates the presence of three newly discovered, immunoreactive 31-amino acid endothelins as well as ETs(1-21), big ET-1 and big ET-3 in the granulocytes of normal human subjects. Reversed-phase HPLC profiles showed that the immunoreactive ETs(1-31), except the unknown compound eluted at 95 min, are identical to authentic ET-1, -2, and -3(1-31). The eluate at 95 min may be a nicked ET-1(1-31) or an oxidation product, although this remains to be proven. Furthermore, it is intriguing that the levels of ET-1 and -3(1-31) in human granulocytes are higher than those of ET-1 and -3(1-21). As similar profile of ET derivative levels was obtained in human lungs (manuscript in preparation). Further studies to compare the distribution of ETs, including ETs(1-31), in various human organs, cells, plasma, and cerebrospinal fluid, and the clinical and pathophysiological significance of the measurements of immunoreactive ETs(1-31) are currently in progress.

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