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CHARACTERIZATION OF ENDOTHELIN SECRETION BY VASCULAR ENDOTHELIAL CELLS

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SUMMARY: **The characterization of mechanisms that regulate ET-LP secretion from bovine adrenal cortical capillary endothelial cells (ACE) in culture was performed by developing radioimmunoassays that distinguish between ET^{1-21} ($AbET^{1-21}$) and ET^{1-39} ($AbET^{1-39}$). The conditioned media (DMEM) content of ET-like immunoreactivity ($ET^{1-21}LI$) increased from 50 to 350 pg/ml over a 24 h period. Addition of 10% calf serum or 0.1% BSA enhanced $ET^{1-21}LI$ release 2-3 fold. Authenticity of $ET^{1-21}LI$ was examined using reversed phase liquid chromatography. All $ET^{1-21}LI$ co-eluted with authentic ET-1. Examination of $ET^{1-39}IR$ by liquid chromatography revealed two peaks of immunoreactivity, one co-eluting with authentic ET^{22-39} and a later running peak co-eluting with authentic ET^{1-39}. Neither $ET^{1-21}LI$ nor $ET^{1-39}LI$ was detected in the extracts of sonicated ACE cells. Treatment of cells with various forms of TGFß significantly augmented $ET^{1-21}LI$ release. These data suggest that ACE secretion of ET-LP *in vitro* occurs spontaneously and can be enhanced by TGFß. Since neither $ET^{1-21}LI$ nor $ET^{1-39}LI$ was detectable in ACE cells it is unlikely that ET-LP are stored prior to their secretion.**

Endothelin$^{1-21}$ (ET^{1-21}), a 21 amino acid containing peptide synthesized and released by vascular endothelial cells, stimulates contraction of vascular (1, 2), bronchial (3-5) and cardiac smooth muscle (6, 7). In addition to its effect on smooth muscle mechanics ET^{1-21} may also act as a growth factor in vascular smooth muscle (8, 9). ET^{1-21} is identical in length, disulfide and C-terminal residues to two other peptides identified using a human genomic DNA library (10). ET^{1-21} as well as a C-terminally extended form, ET^{1-39}, are processed products of proendothelin (1). The

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role of ET^{1-39} as a precursor of ET^{1-21} has not been established, although the primary sequence of ET^{1-21}, ET^{22-39} and ET^{1-39} has been characterized in the conditioned media of endothelial cells in culture (11).

ET^{1-21}-like immunoreactive material ($ET^{1-21}IR$) and $ET^{1-39}IR$-like immunoreactive material ($ET^{1-39}IR$) are released from cultured endothelial cells (1, 11, 12). [Arg^8]vasopressin, angiotensin II, thrombin (11) and $TGF\beta_1$ (13) augment the secretion of these endothelin-like peptides (ET-LP). We have developed radioimmunoassays for ET^{1-21} and ET^{1-39} in an effort to characterize the mechanisms that govern the secretion of these peptides from vascular endothelial cells *in vitro*.

MATERIALS AND METHODS

Materials. ET^{1-21}, ET^{1-21} analogs and fragments, [Tyr^{21},Phe^{31}]ET^{21-39}, ET^{22-39} and sarafotoxin S6b were synthesized by solid phase synthesis (14). ET^{1-39} was purchased from Peptides International, Louisville, KY. Transforming growth factors were purchased from R & D Systems, Minneapolis, MN.

Preparation of ET^{1-21} and ET^{1-39} antiserum. Rabbits were immunized with ET^{1-21} or ET^{31-39} immunogen prepared by coupling the peptide to human α-globulin (Research Plus Laboratories, Inc., Denville, NJ) using glutaraldehyde. Peptide and a four-fold (by weight) excess of carrier protein were dissolved separately in 0.1 M sodium phosphate buffer, pH 7.0, then mixed on a magnetic stirrer. Glutaraldehyde, 25% w/v (Ted Pella) was diluted to 0.375% in phosphate buffer and slowly added with stirring to a final dilution of 0.14%. The reaction was allowed to proceed for 5 h at room temperature and the reaction products dialyzed against 0.9% saline over a 24 h period.

Rabbits were immunized with an emulsion of Freund's complete adjuvant and the prepared conjugate and saline mixed in equal volumes. Each rabbit initially received 2 mg of immunogen and was subsequently boosted at monthly intervals with 1 mg (in 1 ml) distributed between 20 intradermal sites. Animals were bled 10 days after each booster injection. Antisera ($AbET^{1-21}$ or $AbET^{1-39}$) were kept frozen at -20° until the titer was measured by the amount of iodinated ligand bound.

Radioimmunoassays. [^{125}I-Tyr^{13}]ET^{1-21} (^{125}I-ET^{1-21}) was obtained from DuPont NEN Products (Boston, MA). [^{125}I-Tyr^{21},Phe^{31}]ET^{21-39} (^{125}I-ET^{21-39}) was prepared using Chloramine T and purified on a C_{18} reversed phase cartridge. Radioimmunoassays (RIA) were carried out in buffer containing 0.05 M sodium phosphate, 0.15 M sodium chloride, 0.025 M Na_2EDTA, 0.25% BSA (Crystalline, Pentex, Miles) and 0.1% sodium azide at pH 7.4. After addition of 100 μl of standard or test sample, 100 μl of antibody, $AbET^{1-21}$ or $AbET^{1-39}$, at a final dilution of 1:160,000 or 1:60,000, respectively, was added to borosilicate tubes (4°). ET^{1-21} or [Tyr^{21},Phe^{31}]ET^{21-39} served as standards when using either $AbEt^{1-21}$ or $AbET^{1-39}$, respectively. Tubes were mixed and 10,000 cpm of iodinated ligand (^{125}I-ET^{1-21} or ^{125}I-ET^{21-39}) in 100 μl of RIA buffer was added and allowed to incubate (4°) overnight. Separation of free from bound tracer was accomplished by adding 100 μl of normal rabbit serum as co-precipitant (1:200), 100 μl of goat anti-rabbit serum (1:40) and 500 μl of 10% polyethylene glycol. After incubation at 4° for 15 min and centrifuging at 3,000 rpm for 20 min, supernatants were aspirated and the pellets counted in a Micromedic gamma counter.

Endothelial cell culture. Bovine adrenal cortex capillary endothelial cells were prepared, cloned and maintained as described (15, 16). Cells were grown in 24 well dishes to confluence in HEPES (25 mM) buffered Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% calf serum, 1 ng/ml basic fibroblast growth factor, fungizone and gentamycin. At confluence, the cells were washed twice with 0.01 M NaH_2PO_4, pH 7.2, containing 0.15 M NaCl (PBS) and the culture medium was replaced with DMEM containing various agents. The culture medium was collected at various times, the cells lysed by sonication in PBS and $ET^{1-21}IR$ or $ET^{1-39}IR$ determined.

Chromatography. Reversed phase liquid chromatography (RP-HPLC) was performed using a Beckman 450 Data/System controller, Model 165 variable wavelength detector and two 114M pumps. Solvent A was 0.1% trifluoroacetic acid (TFA, Pierce) in chromatographic grade water; solvent B was 80% CH_3CN in 0.1% TFA. Samples were partially purified using C_{18} reversed phase cartridges [wash solution: 2 volumes 1% formic acid adjusted to pH 3 with triethyl amine (TEAF); eluant: 2 volumes 75% acetonitrile/25% TEAF] and injected (100 μl loop) onto a Vydac C_{18} 5 μm reversed phase column. A linear gradient from 5 to 70 % acetonitrile was applied over a period of 45 min (flow rate: 1 ml/min) and 1 ml fractions collected. After removal of solvent under reduced pressure, tube contents were assayed for $ET^{1-21}IR$ or $ET^{1-39}IR$.

RESULTS

AbET$^{1-21}$ specificity was characterized by assessing the ability of ET$^{1-21}$ and a variety of analogs to displace ^{125}I-ET$^{1-21}$ from antibody (Fig. 1). [D-Cys]ET$^{1-21}$ was nearly as effective as ET$^{1-21}$ in displacing bound ligand, EC_{50} = 70 and 30 fmoles, respectively; endothelin-3 (ET-3) was equipotent with ET$^{1-21}$. Linearization of ET$^{1-21}$ through oxidation of cysteine residues at positions 1,3,11,15 or amidocarboxymethylation of these residues [Cys(ACM)] produced compounds that could effectively displace ^{125}I-ET$^{1-21}$, EC_{50} = 130 and 90 fmoles, respectively. Moreover [Cys(ACM)^{15},desHis^{16}]-ET$^{15-21}$ could displace ^{125}I-ET$^{1-21}$ with an EC_{50} of 200 fmoles. Peptides with C-terminal substitutions such as [D-TRP^{21}]ET$^{1-21}$ and sarafotoxin S6b displaced ^{125}I-ET$^{1-21}$ poorly, EC_{50} = 2.5 and 6 pmoles, respectively. [Arg^{8}]vasopressin, calcitonin gene related peptide and somatostatin-28 were ineffective in displacing ^{125}I-ET$^{1-21}$, EC_{50} > 20 nmoles. These data portend that the antibody is highly specific for the C-terminal portion of ET$^{1-21}$.

AbET$^{1-39}$ specificity was characterized by assessing the ability of ET$^{1-39}$ and various fragments to displace ^{125}I-ET$^{1-39}$ from antibody (data not shown). ^{125}I-ET$^{21-39}$ was displaced from AbET$^{1-39}$ by ET$^{1-39}$, ET$^{31-39}$ and [Tyr^{21},Phe^{31}]ET$^{21-39}$ with equal efficacy, EC_{50} = 350 fmoles; ET$^{1-21}$ did not displace ^{125}I-ET$^{21-39}$, EC_{50} > 2 nmoles. [Arg^{8}]vasopressin was ineffective in displacing ^{125}I-ET$^{1-39}$. Thus AbET$^{1-39}$ will detect both ET$^{22-39}$ and ET$^{1-39}$ but not ET$^{1-21}$.

Optimization of culture conditions was achieved by measuring ET$^{1-21}$IR in the conditioned media of confluent bovine adrenocortical capillary endothelial cells at various times after incubation in PBS, DMEM, DMEM plus 0.1% BSA and DMEM plus 10% calf serum (Fig. 2). The ET$^{1-21}$IR content of cells incubated in DMEM increased linearly with time from 50 to 350 pg/ml over a 24 hour period. If DMEM was supplemented with either 0.1% BSA or 10% calf serum, the release was likewise linear but ET$^{1-21}$IR secretion was enhanced 2-3 fold. The increase in ET$^{1-21}$IR was linear up to 48 h when cells were exposed to DMEM supplemented with 10% calf serum (Fig. 2 *INSET*). No ET$^{1-21}$IR was measurable in the conditioned media of cells incubated in PBS (Fig. 2). In contrast to the presence of ET$^{1-21}$IR in conditioned media neither ET$^{1-21}$IR nor ET$^{1-39}$IR was detected in sonicated extracts of endothelial cells [ET$^{1-21}$IR (< 15 pg/10^5 cells) or ET$^{1-39}$IR (< 90 pg/10^5 cells)].

The identity of ET$^{1-21}$IR in culture media was examined using RP-HPLC. Partial purification of conditioned media obtained after a 12 h incubation in DMEM plus 10% calf serum using reversed

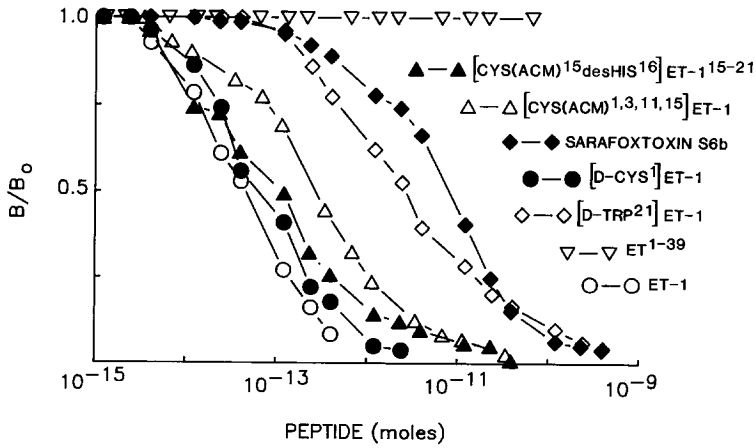

Fig 1: Displacement of ^{125}I-ET$^{1-21}$ by ET$^{1-21}$ and various ET analogs.

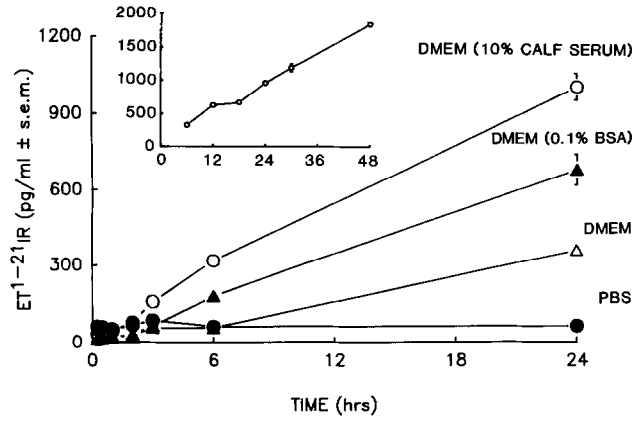

Fig 2: $ET^{1-21}IR$ in endothelial cell culture media after incubation (37°C) for various times. *INSET* Extended time course for secretion of $ET^{1-21}IR$ in DMEM with 10% calf serum.

phase extraction followed by RP-HPLC revealed the presence of an immunoreactive peak co-eluting with authentic ET^{1-21} (Fig. 3). Re-chromatography of this peak in a second gradient system produced a peak of immunoreactivity that co-eluted with authentic ET^{1-21} (data not shown). Analysis of $ET^{1-39}IR$ obtained from conditioned media 18 h after incubation in DMEM plus 0.1% BSA by RP-HPLC demonstrated the presence of two peaks, one co-eluting with authentic ET^{1-39} and an earlier running peak co-eluting with authentic ET^{22-39} (Fig. 4).

The influence of various forms of TGFß on $ET^{1-21}IR$ secretion from endothelial cells was examined at varying times (Table I). These growth factors increased $ET^{1-21}IR$ presence in culture from 3-10 fold. Cells maintained in 10 % calf serum released greater amounts of $ET^{1-21}IR$ in the medium than those kept in 0.1 % BSA.

The temporal co-secretion of $ET^{1-21}IR$ and $ET^{1-39}IR$ was studied using $TGFß_1$. Examination of $ET^{1-39}IR$ and $ET^{1-21}IR$ secretion from cultured cells maintained in 0.1 % BSA revealed a parallel

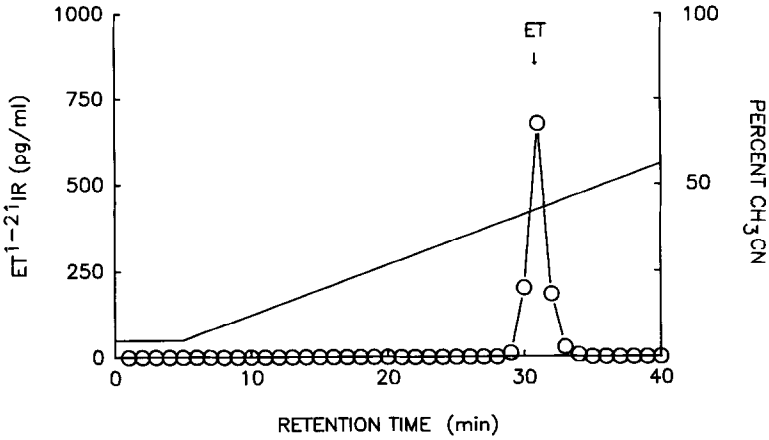

Fig 3: $ET^{1-21}IR$ in fractions after RP-HPLC of partially purified endothelial cell culture medium (DMEM with 10% calf serum). A linear gradient from 5 to 70 % acetonitrile was applied over a period of 45 min (flow rate: 1 ml/min) and 1 ml fractions collected.

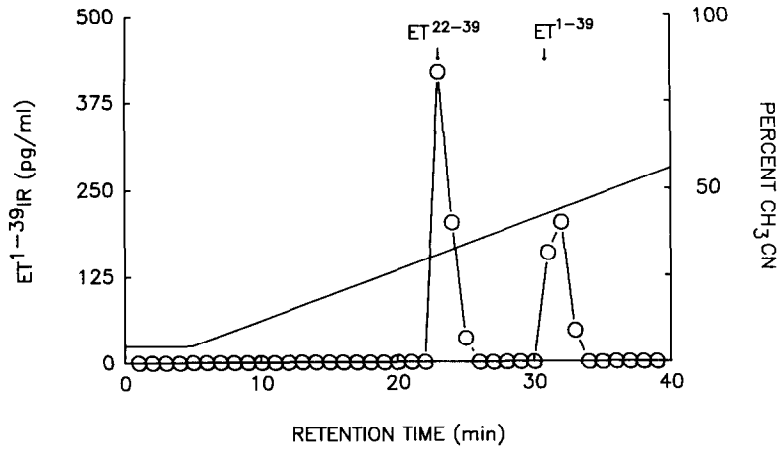

Fig 4: $ET^{1-39}IR$ in fractions after RP-HPLC of partially purified endothelial cell culture medium (DMEM supplemented with 0.1% BSA). A linear gradient from 5 to 70 % acetonitrile was applied over a period of 45 min (flow rate: 1 ml/min) and 1 ml fractions collected.

increase with time (Fig. 5). A significant augmentation in secretion of both forms of immunoreactive material occurred when TGFß, (10 ng) was present in the media.

DISCUSSION

Two specific antisera have been developed for the characterization of secretion of ET-LP from cultured endothelial cells. $AbET^{1-21}$ is specific for the C-terminal portion of ET^{1-21} since substitution of D-Trp at position 21 resulted in a significant (100 fold) loss in ability to displace bound ligand (Fig. 1). Substitution at the N-terminal portion of ET^{1-21} or linearization of the molecule results in peptides that are only 3-4 fold less effective in displacing ^{125}I-ET^{1-21} than ET^{1-21}. Moreover sarafotoxin S6b which differs at several positions from ET^{1-21} including positions 17 and 19 poorly displaces bound ligand (200 fold less effective). Thus this antibody is a valuable agent in the characterization of secretion of ET-LP from endothelial cells.

TABLE I

Effects of Different TGFßs on Endothelin Secretion From Cultured Vascular Endothelial Cells

TREATMENT	TIME (h)			
	5	18	5	18
	0.1 % BSA in DMEM		10 % CS in DMEM	
Control	54 ± 5	196 ± 13	74 ± 16	590 ± 59
$TGFß_1$ (10 ng)	87 ± 4	628 ± 46	106 ± 13	1154 ± 72
$TGFß_{1,2}$ (10 ng)	138 ± 4	470 ± 20	133 ± 4	1230 ± 60
$TGFß_2$ (10 ng)	93 ± 8	609 ± 91	140 ± 7	--

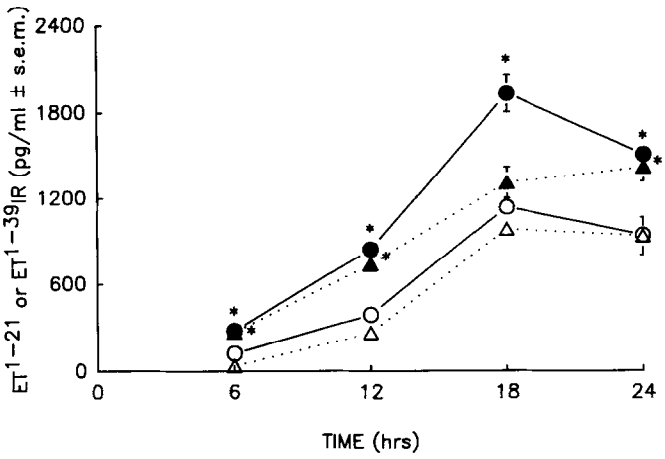

Fig 5: $ET^{1-21}IR$ (solid line) and $ET^{1-39}IR$ (dotted line) in endothelial cell culture media after incubation (37°) with 10 ng of $TGF\beta_1$ (closed symbols) for various times.

$AbET^{1-39}$ recognizes the C-terminal portion of ET^{1-39} since ET^{31-39} and $[Tyr^{21}][Phe^{31}]ET^{22-39}$ are as effective as ET^{1-39} in displacing bound ligand. ET^{1-21} does not displace bound ligand from $AbET^{1-39}$. The development of this antibody provides an additional powerful tool for the characterization of ET-LP secretion.

Incubation of confluent bovine adrenocortical capillary endothelial cells in DMEM results in the spontaneous release of $ET^{1-21}IR$ from 50 to 350 pg/ml over 24 h (Fig. 2). Supplementation of the medium with either 0.1 % BSA or 10 % calf serum enhanced the release 2-3 fold. Secretion increased in a linear fashion over a 24 h period. Interestingly, $ET^{1-21}IR$ secretion was not detectable when cells were incubated in PBS. These data suggest that DMEM contains an essential factor required for secretion of $ET^{1-21}IR$. Analysis of sonicated extracts of endothelial cells for $ET^{1-21}IR$ and $ET^{1-39}IR$ did not reveal the presence of ET-LP. This fact coupled with the accumulation of $ET^{1-21}IR$ in culture media supports the notion that ET^{1-21} is not released from a cellular storage pool. Thus is appears that ET-LP are synthesized and subsequently secreted from endothelial cells. Addition of any of the various forms of TGFß to cells incubated in DMEM supplemented with either 0.1 % BSA or 10 % calf serum significantly augmented the secretion of $ET^{1-21}IR$ after 18 h (Table I and Fig. 5). Examination of conditioned media for the co-secretion of ET-LP revealed that $ET^{1-21}IR$ and $ET^{1-39}IR$ increased in a parallel fashion. This pattern of increase was also observed after the addition of TGFß1 to the medium (Fig. 5).

Analysis of the $ET^{1-21}IR$ appearing in conditioned medium by RP-HPLC revealed a single immunoreactive peak that co-eluted with authentic ET^{1-21} (Fig. 3). Chromatography in a second gradient system confirmed the identity of ET^{1-21}. Analysis of conditioned medium for $ET^{1-39}IR$ divulged the presence of two immunoreactive peaks, one co-eluting with authentic ET^{22-39} and one with authentic ET^{1-39} (Fig. 4). These data provide further support for the contention that the ET-LP appearing in conditioned media does not arise from a cellular storage site since ET^{1-39} is detectable in the culture media but not in endothelial cell extracts.

These data demonstrate that endothelial cell secretion of ET-LP *in vitro* occurs spontaneously and can be enhanced by $TGF\beta_1$. Since neither $ET^{1-21}IR$ nor $ET^{1-39}IR$ were detectable in endothelial cells it is unlikely that ET^{1-21} is stored prior to secretion. The presence of ET^{22-39} in the culture

medium suggests that the conversion of ET^{1-39} to ET^{1-21} and ET^{22-39} occurs at the level of the cellular membrane or in the culture medium. Whether this pattern of secretion is representative of endothelial cells *in vivo* remains to be determined.

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