Volume 239, number 1, 13–17 FEB 06419 October 1988

Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells

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Received 6 August 1988; revised version received 30 August 1988

Binding of a novel endothelium-derived vasoconstrictor endothelin (ET) and the regulation of its receptor were studied in cultured rat vascular smooth muscle cells. ^{125}I-labeled-ET bound to the cells was resistant to acid extraction and the majority of the acid-resistant compartment was extractable with chloroform/methanol with minimal degradation. Autoradiographic studies using electron microscopy revealed that the grains were predominantly localized in the plasma membranes, but some were adjacent to and within the lysosome. Pretreatment with ET resulted in a substantial reduction of ET receptor number without changing its binding affinity. ET-induced increase in cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) was absent or attenuated in the ET-pretreated cells. These data suggest that tight association of ET with its receptor is due to a strong interaction of its hydrophobic domain with the membrane lipids and/or its internalization within cells and that down-regulation of ET receptor is functionally linked to decreased ET-induced $[Ca^{2+}]_i$ response.

Endothelin; Receptor binding; Receptor down-regulation; cytosolic free Ca^{2+}; (Vascular smooth muscle cell)

1. INTRODUCTION

Endothelin (ET) is a novel vasoconstrictor peptide derived from cultured porcine endothelium, and consists of 21-amino-acid residues [1]. It induces a potent and sustained vasoconstriction of a variety of blood vessels from many species. This biological action is dependent on extracellular Ca^{2+}, suggesting that Ca^{2+} influx is a prerequisite for its action.

Using cultured rat vascular smooth muscle cells (VSMCs), we have recently demonstrated the presence of specific receptors for ^{125}I-labeled-porcine (p) ET through which pET induces a large increase in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in fura-2-loaded cells [2]. In the present study, we have attempted to elucidate the mechanism of ET binding and the down regulation of its receptor in cultured rat VSMCs.

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2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic pET was obtained from Peptide Institute (Osaka, Japan). Radiolabeled pET was prepared by iodination using ^{125}INa (New England Nuclear, Boston, MA) and lactoperoxidase (Sigma Chemicals, St. Louis, MO), and purified by Sep-Pak C_{18} cartridge (Millipore-Waters, Milford, MA) using 70% acetonitrile/0.1% trifluoroacetic acid as an eluent as described in [2]: specific activity was 400–500 Ci/mmol. VSMCs were obtained from the thoracic aorta of adult Wistar rats by the explant method [3] and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Flow Laboratories, McLean, VA) as reported [4]. Subcultured cells (5–20th passages) were used in the experiments.

2.2. *Binding experiments*

Confluent (5×10^5) cells were usually incubated with 1 nM ^{125}I-pET at 37°C for 60 min in Hanks' balanced salt solution containing 0.1% bovine serum albumin [2] unless otherwise stated. After completion, cells were extensively washed, solubilized in 0.5 N NaOH, and the cell-bound radioactivity was determined. Specific binding was obtained by subtracting nonspecific binding in the presence of an excess (4×10^{-7} M) unlabeled pET from total binding.

2.3. *Compartmentalization of cell-bound ^{125}I-pET*

To determine the compartmentalization of ET bound to the

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00145793/88/$3.50

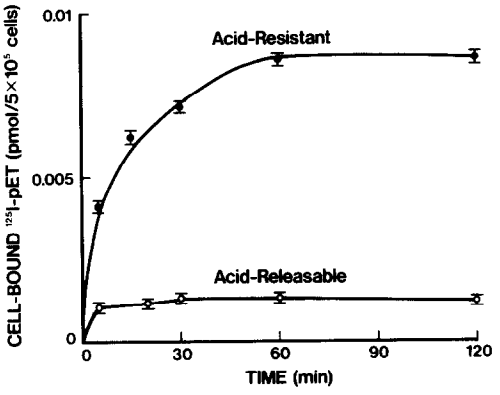

Fig.1. Binding of ^{125}I-pET to rat VSMCs as a function of time. Cells were incubated with ^{125}I-pET at 37°C for indicated times. Both surface-bound ^{125}I-pET removed with acid treatment (○, acid-releasable) and the remaining cell-bound ^{125}I-pET solubilized with NaOH (½, acid-resistant) were determined. Each point is the mean of triplicate dishes: bars show SE. Specific binding was 80% of total binding.

cells, VSMCs were incubated with ^{125}I-pET at 37°C and the cell-bound ligand was removed by treating the cells with 0.2 M acetic acid, pH 2.5, containing 0.5 M NaCl, at 4°C for 6 min that extracts only surface-bound ligand without damaging the receptors or extracting internalized ligand [5]. The acid-resistant compartment was extracted from chloroform/methanol (2:1) to separate lipid- from water-soluble fractions.

2.4. *Gel exclusion chromatography*

To analyze the nature of ^{125}I-pET during incubation, aliquots of the incubation medium, acid extract and cell extract were applied to a Sephadex G-50 fine resin (Pharmacia, Uppsala, Sweden) column (30 × 8 cm) equilibrated with 0.1 M ammonium acetate, pH 7.4: 1.2-ml fractions each were collected and radioactivity was measured.

2.5. *Autoradiographic analysis*

After incubation with ^{125}I-pET at 37°C for 60 min, the cells were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, at 4°C for 10 min and processed for autoradiographic analysis by electron microscopy (EM) using Hitachi H-600 as previously reported [6].

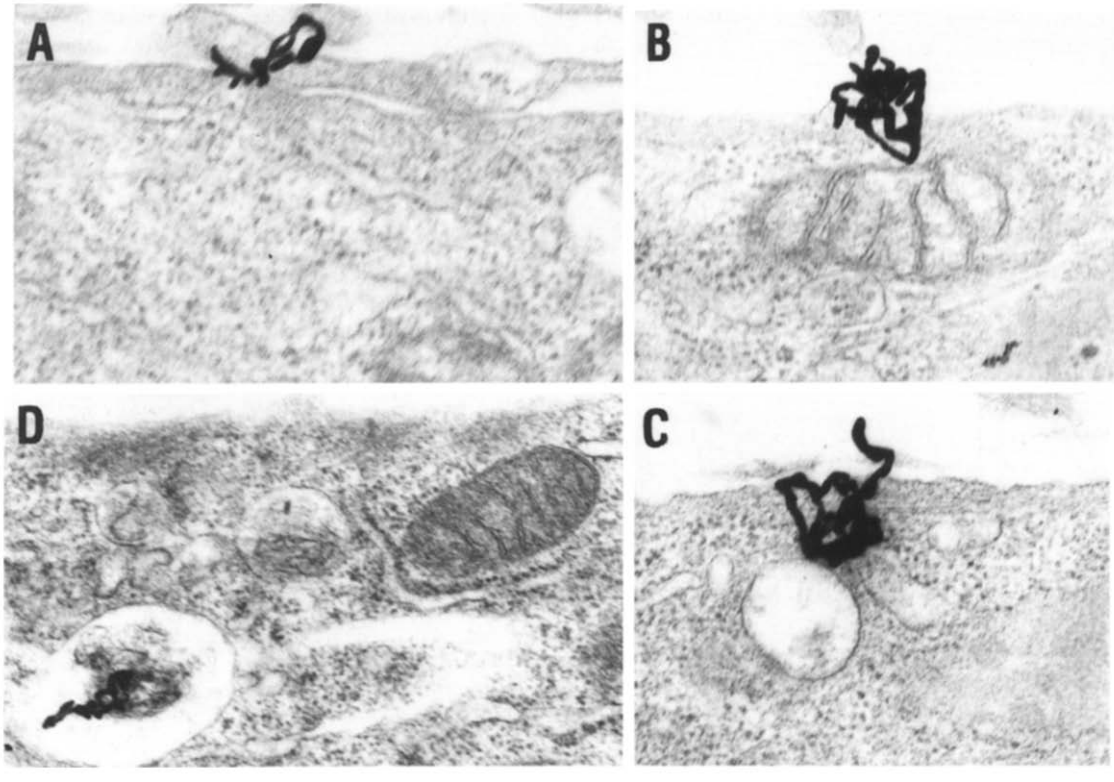

Fig.2. Selected EM images of rat VSMCs with developed autoradiographic grains. Cells were incubated with ^{125}I-pET at 37°C for 60 min and processed for EM autoradiography. Original magnification (A,D, × 30000; B,C, × 40000).

2.6. *Measurement of* $[Ca^{2+}]_i$

After trypsinization and incubation with 4 μM fura-2 acetoxymethyl ester (Dojin Chemical, Kumamoto, Japan) at 37°C for 20 min in Hepes-buffered physiological salt solution, pH 7.4, fluorescence of fura-2-loaded suspended cells (2×10^6 cells) was measured with a Hitachi MDF-4 spectrofluorimeter as described [2] and $[Ca^{2+}]_i$ values were calculated according to the method of Grykiewicz et al. [7].

2.7. *Down regulation of ET receptor*

To investigate whether vascular ET receptor is down regulated, cells were incubated in the absence and presence of pET (10^{-9} and 10^{-8} M) in serum-free DMEM at 37°C for 24 h. After incubation, cells were extensively washed with ice-cold DMEM to remove the unbound pET and reincubated in fresh DMEM at 37°C for 90 min to permit full recovery of the receptor-binding capacity. The cells were then subjected to saturable binding experiments and determination of $[Ca^{2+}]_i$ after fura-2-loading.

3. RESULTS

As shown in fig.1, ^{125}I-pET time-dependently bound to rat VSMCs at 37°C, reaching an apparent equilibrium after 60 min. Only 10–25% of surface-bound ^{125}I-pET was dissociable from the cells with dilute acid, while the majority of cell-bound ligand was transferred to the acid-resistant compartment; 91 ± 2% of the ligand in the acid-resistant compartment was extractable with chloroform/methanol. Gel filtration of incubation medium, acid extract and cell extract gave almost identical elution profiles of ^{125}I-pET with the major component (>90%) eluting at the position of standard pET and a minor one eluting in the void

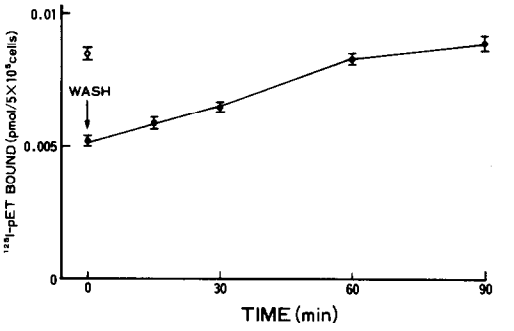

Fig.3. Recovery of ^{125}I-pET-binding capacity after pretreatment with unlabeled pET. After pretreatment with 10^{-9} M unlabeled pET, cells were washed and reincubated in pET-free medium for indicated times and ^{125}I-pET-binding activity was determined. Binding capacity of the control cells incubated in the absence of pET is shown by open circle. Each point is the mean of triplicate dishes: bars show SE.

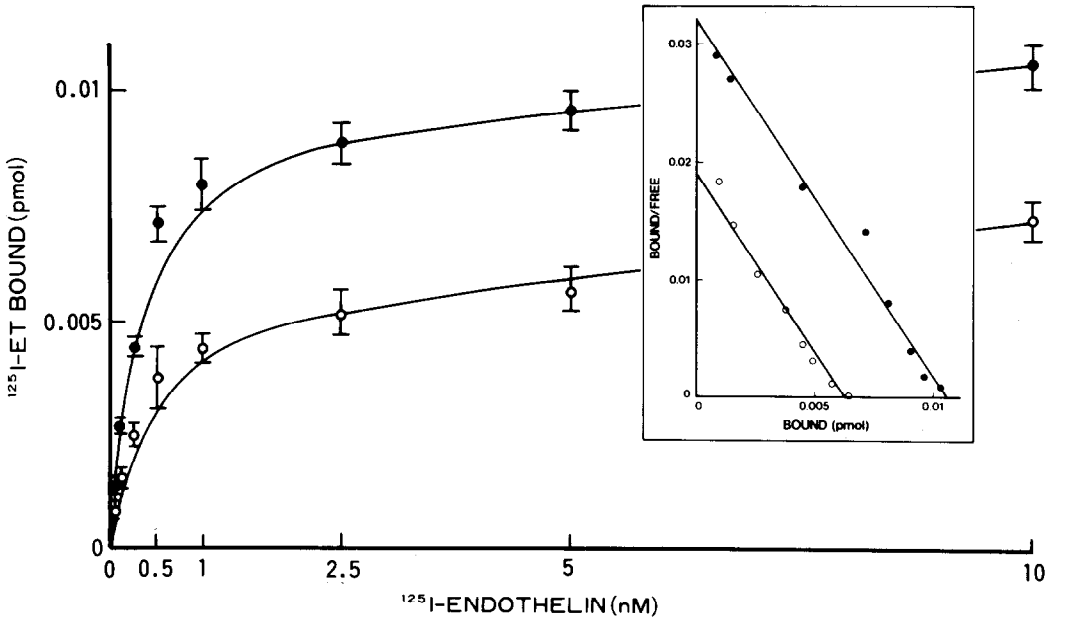

Fig.4. Saturable binding of ^{125}I-pET to rat VSMCs after long-term treatment with or without unlabeled pET. Cells were incubated at 37°C for 24 h in the absence (½) and presence (○) of 10^{-9} M unlabeled pET, washed and reincubated in pET-free medium for 90 min, after which saturable binding experiment was performed as described in section 2. Each point is the mean of three experiments; bars show SE. (Inset) Scatchard plots of binding data.

volume, possibly representing the aggregated and/or noncovalently-bound form: no obvious component(s) eluting later than pET was (were) observed (not shown).

Autoradiographic studies using EM revealed that the silver grains were predominantly associated with the plasma membranes (fig.2A) and penetrated through the membrane into the cytoplasm (fig.2B), some of which were found to be located adjacent to (fig.2C) and within (fig.2D) lysosome-like structures.

When VSMCs were incubated with 10^{-9} M unlabeled pET at 37°C for 60 min, followed by washing and reincubation in fresh medium, the receptor-binding activity of ^{125}I-pET, although significantly decreased at the beginning, gradually restored and reached that of untreated cells after 90 min (fig.3), suggesting complete recovery of receptor-binding capacity. Pretreatment with 10^{-9} M unlabeled pET for 24 h resulted in a marked decrease in ^{125}I-pET-binding capacity (fig.4). Scatchard analysis showed a substantial reduction of total ET receptor number in pET-treated cells (7200 sites/cell) compared to that of non-treated cells (13 000 sites/cell) without changing its binding affinity (K_d: 3×10^{-10} M) in both cells, indicating receptor down-regulation. Similar reduction in ^{125}I-pET-binding capacity was induced with 10^{-8} M unlabeled pET (not shown).

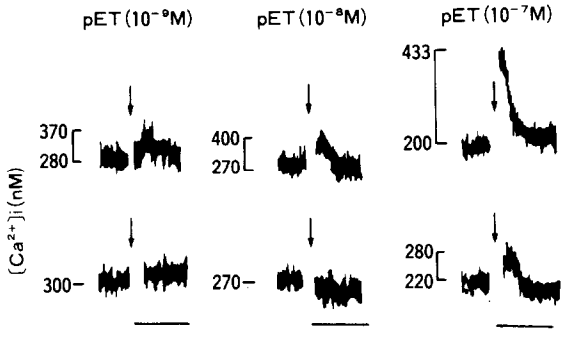

Fig.5. Effect of pET on changes of $[Ca^{2+}]_i$ after long-term treatment with or without unlabeled pET. After pretreatment with or without 10^{-9} M unlabeled pET for 24 h, cells were treated the same as in fig.4 and loaded with fura-2 as described in section 2. Fura-2-loaded cell suspensions were then challenged with various doses (10^{-9}–10^{-7} M) of pET as indicated by arrows in non-treated cells (upper panel) and in pET-treated cells (lower panel). Calculated values of $[Ca^{2+}]_i$ are shown on the ordinates. 1 min interval is underlined.

The effect of various doses (10^{-9}–10^{-7} M) of pET on changes of $[Ca^{2+}]_i$ in fura-2-loaded cells pretreated with or without 10^{-9} M pET is shown in fig.5. pET dose-dependently induced transient increases in $[Ca^{2+}]_i$ in non-treated cells. In pET-treated cells, however, lower doses (10^{-9} and 10^{-8} M) of pET failed to increase $[Ca^{2+}]_i$, while pET in high doses (10^{-7} M) only increased $[Ca^{2+}]_i$ to a lesser extent compared to that in non-treated cells.

4. DISCUSSION

ET is a novel vasoconstrictive peptide derived from endothelium [1] which acts directly on VSMCs by binding to its specific membrane-bound receptors to induce Ca^{2+} influx, possibly through the voltage-dependent Ca^{2+} channels, thereby leading to vasoconstriction [2].

The pET-induced vasoconstriction is long-acting and characteristically difficult to washout as demonstrated in isolated vascular strips [1] as well as in cultured rat VSMCs [2]. The present study clearly shows that ^{125}I-pET bound to the cells is resistant to dissociation even after acid treatment that only extracts surface-bound ligands [5] and the majority of cell-bound ^{125}I-pET in the acid-resistant compartment was extractable with chloroform/methanol. Based on the hydrophilic nature of the ET molecule, our data raise the possibility that ET bound to its membrane receptor may interact tightly with membrane lipids, possibly phospholipids in the surrounding lipid environment. Autoradiographic studies using EM demonstrate the exclusive localization of silver grains associated with plasma membranes. It should be noted that some grains were in close contact with or trapped within the lysosome-like structures, suggesting internalization of ET within the cell and subsequent sequestration by lysosome. Therefore, internalized ET may partly represent a fraction of the acid-resistant compartment.

The present study also shows that the unbound ^{125}I-pET in the medium as well as the cell-bound forms in both acid-releasable and -resistant compartments appear to be intact as assessed by gel filtration and their ability to rebind to fresh cells (unpublished). In fact, several proteolytic enzyme inhibitors (phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, captopril, leupeptin) failed to af-

fect the ^{125}I-pET binding, while lysosomotrophic compounds (chloroquin, ammonium chloride) slightly increased the binding (unpublished). Taken together, it is suggested that cell-mediated degradation and/or inactivation of ET occurs little, if any, during incubation.

Our study further demonstrates that long-term exposure of pET leads to a substantial reduction of total ET receptor number without affecting its binding affinity, indicating the down regulation of vascular ET receptor. Concomitantly, pretreatment with pET results in almost absent or attenuated increases in $[Ca^{2+}]_i$ upon subsequent stimulation with pET, suggesting that the down-regulated ET receptors are functionally linked to decreased ET-induced $[Ca^{2+}]_i$ response in VSMCs.

Acknowledgements: We thank Drs S. Takata, Y. Takagi and Y. Fukuda for cooperation, Drs T.X. Watanabe and S. Sakakibara for helpful discussion, and Ms M. Fukushima for technical assistance. This study was supported in part by Research Grants from the Ministry of Health and Welfare (62A-1, 63C-1), and from the Ministry of Education, Culture and Science (62570530), Japan.

REFERENCES

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagai, S., Nakajima, K. and Sakakibara, S. (1988) Biochem. Biophys. Res. Commun., in press.
[3] Ross, R. (1971) J. Cell. Biol. 50, 172–186.
[4] Hirata, Y., Tomita, M., Yoshimi, H. and Ikeda, M. (1984) Biochem. Biophys. Res. Commun. 125, 562–568.
[5] Haigler, H.T., Maxfield, F.R., Willingham, M.C. and Pastan, I. (1980) J. Biol. Chem. 225, 1239–1241.
[6] Hirata, Y., Takata, S., Tomita, M. and Takaichi, S. (1985) Biochem. Biophys. Res. Commun. 132, 976–984.
[7] Grykiewicz, G., Poenie, M. and Tsien, M.P. (1985) J. Biol. Chem. 260, 3440–3450.