Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 228-234

RECEPTOR BINDING ACTIVITY AND CYTOSOLIC FREE CALCIUM RESPONSE BY SYNTHETIC ENDOTHELIN ANALOGS IN CULTURED RAT VASCULAR SMOOTH MUSCLE CELLS

Y. Hirata[1*], H. Yoshimi[2], T. Emori[1], M. Shichiri[1], F. Marumo[1], T.X. Watanabe[3], S. Kumagaye[3], K. Nakajima[3], T. Kimura[3] and S. Sakakibara[3]

[1]Department of Medicine, Tokyo Medical and Dental University, Tokyo 113, Japan

[2]Department of Medicine, National Cardiovascular Center, Osaka 565, Japan

[3]Peptide Institute Inc., Protein Research Foundation, Osaka 562, Japan

Received March 6, 1989

Summary: Using a variety of synthetic analogs of porcine endothelin (pET), we have studied the effects of these analogs on receptor binding activity and cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in cultured rat vascular smooth muscle cells (VSMC). Removal of C-terminal Trp^{21} residue, truncated derivatives pET(1-15) and (16-21), substitution of disulfide bond, Cys(3-11) or Cys(1-15), by Cys (Acm), all resulted in a complete loss of receptor binding activity and $[Ca^{2+}]_i$ response, while N-terminal elongation of Lys-Arg residues, but not oxidation of Met^7 residue, decreased receptor binding activity and $[Ca^{2+}]_i$ response. $[Cys^{1-15},Cys^{3-11}]$pET was far more potent than $[Cys^{1-11}, Cys^{3-15}]$pET in receptor binding and $[Ca^{2+}]_i$ response. These data indicate that the C-terminal Trp^{21} as well as the proper double cyclic structure formed by the intramolecular disulfide bonds of the pET molecule are essential for receptor binding and subsequent $[Ca^{2+}]_i$ increase in rat VSMC.

Porcine endothelin (pET), a novel endothelium-derived vasoconstrictor peptide, comprises 21 amino-acid residues with two intramolecular disulfide linkages (1). pET induces a potent and sustained vasoconstriction of a variety of blood vessels from many species, of which effect is dependent on extracellular Ca^{2+} (1). The cDNA cloning for human (h) and rat (r) ET has revealed

* All correspondence should be addressed to: Yukio Hirata, M.D., The Second Department of Internal Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113, Japan.

0006-291X/89 $1.50

that hET is identical to pET (2), and rET is similar, but distinct from pET (3). Interestingly, the ET molecules among these species share the two disulfide bonds and the C-terminal hydrophobic region in common, suggesting the importance of these domains in interaction with its receptor.

We have recently demonstrated the presence of specific binding sites for pET in cultured rat vascular smooth muscle cells (VSMC) through which pET induces a profound increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (4). Therefore, the present study was designed to characterize the structural moieties of the pET molecule essential for receptor binding and $[Ca^{2+}]_i$ response in cultured rat VSMC using a variety of synthetic pET analogs.

MATERIALS AND METHODS

Peptides

pET and related peptides were synthesized by solid-phase method and purified by ion-exchange chromatography on DEAE-cellulose and reverse-phase HPLC as described (5). The homogeneity of the final products was confirmed by analytical HPLC and amino-acid analysis.

Binding experiments

Rat aortic VSMCs were cultured and used in the experiments as previously described (6). Binding study was performed essentially in the same manner as recently reported (4). In brief, confluent ($5x10^5$) cells were incubated at 37°C for 60 min with $2.5x10^{-11}$ M ^{125}I-labeled-pET (Amersham Japan, specific activity: 2000 Ci/mmol) in the absence and presence of pET and related peptides. After completion, the cell-bound radioactivity was determined; specific binding was defined as total binding minus nonspecific binding in the presence of $4x10^{-7}$M unlabeled pET.

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

Fluorescence of fura-2-loaded cell suspensions was spectrofluorimetrically measured as described (4) and $[Ca^{2+}]_i$ values were calculated by the formulas described by Grykiewicz et al. (7).

RESULTS

Competitive binding study by synthetic pET and related peptides is shown in Fig. 1. Unlabeled pET(1-21) competitively inhibited the binding of ^{125}I-labeled-pET to rat VSMC with the approximate concentration of half-maximal inhibition (IC_{50}) of

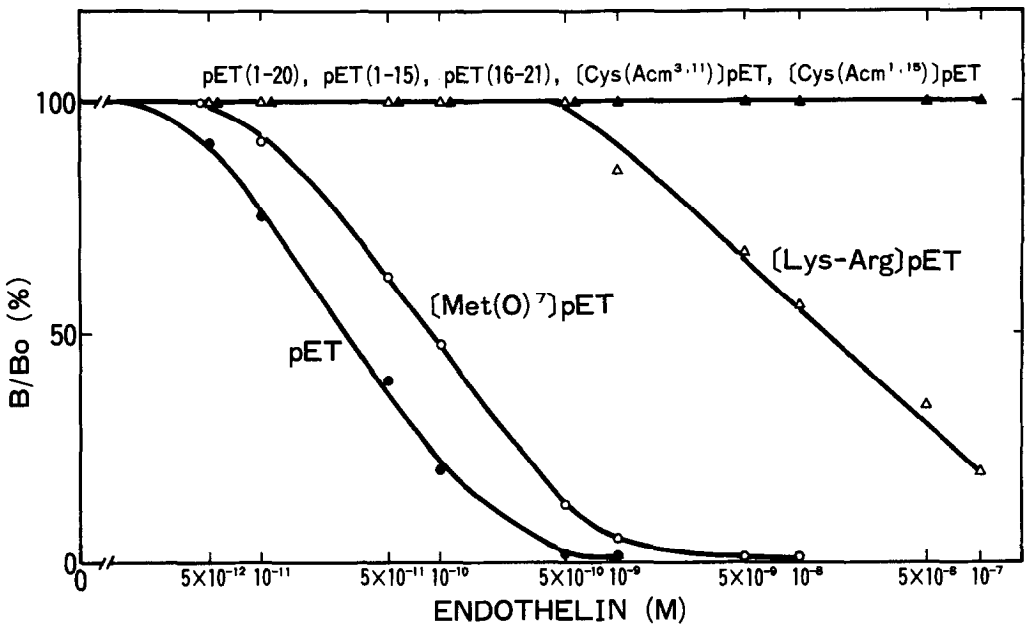

Fig. 1. Competitive binding of ^{125}I-pET to rat VSMC by pET and related peptides.
Confluent cells were incubated at 37°C for 60 min with 2.5×10^{-11} M ^{125}I-pET in the absence and presence of pET and related peptides in concentrations as indicated. Specific binding was 80% of total binding; each point is the mean of two experiments.

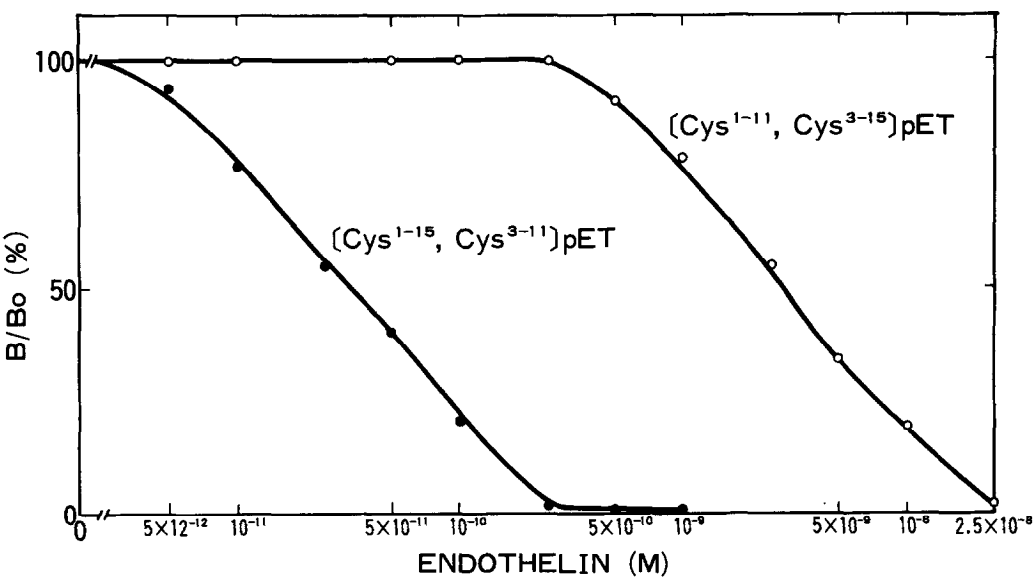

Fig. 2. Competitive binding of ^{125}I-pET to rat VSMC by pET analogs in rat VSMC.
Confluent cells were incubated at 37°C for 60 min with 2.5×10^{-11} M ^{125}I-pET in the absence and presence of [Cys$^{1-15}$,Cys$^{3-11}$]pET (●) and [Cys$^{1-11}$,Cys$^{3-15}$]pET (o) in concentrations as indicated. Specific binding was 82% of total binding; each point is the mean of two experiments.

$3x10^{-11}$ M, while [Met(0)^7]pET was less potent and [Lys-Arg]pET was far less pontent than pET with IC_{50} of 10^{-10} M and $2x10^{-8}$ M, respectively. In contrast, pET(1-20), pET(1-15), pET(16-21), [Cys(Acm)^{3,11}]- and [Cys(Acm)^{1,15}]-pET were ineffective in inhibiting the binding of ^{125}I-pET in concentrations up to 10^{-7} M. [Cys$^{1-15}$,Cys$^{3-11}$]pET was about 100-fold more potent than [Cys$^{1-11}$,Cys$^{3-15}$]pET in displacing ^{125}I-pET from its binding sites (Fig. 2).

The effects of pET and related peptides (10^{-7} M) on increasing $[Ca^{2+}]_i$ in fura-2-loaded VSMC are compared (Fig. 3). [Lys-Arg]pET was less potent, but [Met(0)^7]pET was almost as

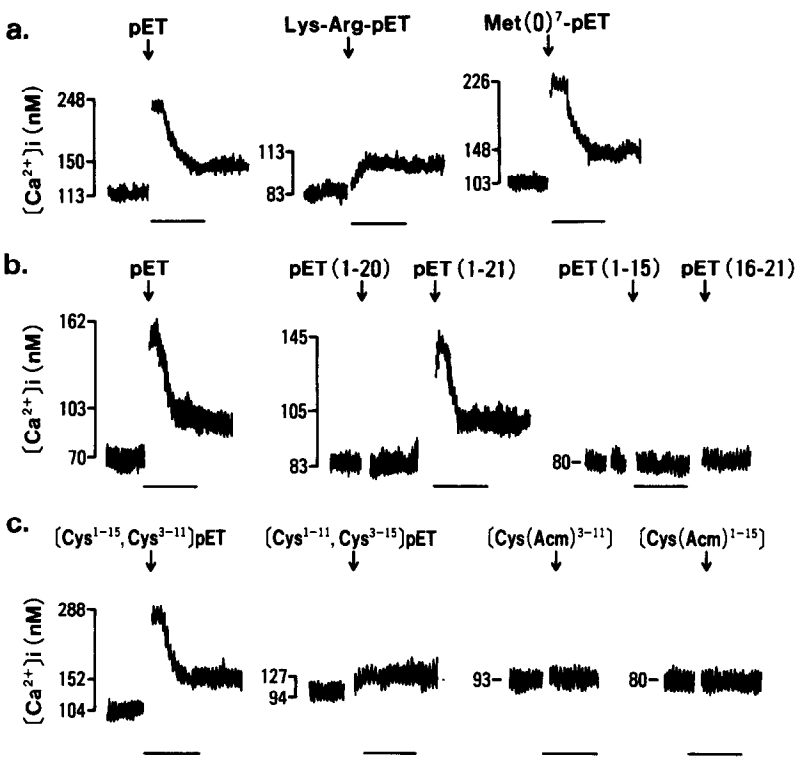

Fig. 3. Ca^{2+}-fura-2 fluorescence by pET and related peptides in rat VSMC.
Fura-2-loaded cell suspensions were challenged with 10^{-7} M each of (a) pET, [Lys-Arg]pET and [Met(0)^7]pET, (b) pET(1-21), pET(1-20) following by pET(1-21), pET(1-15) and pET(16-21), (c) [Cys$^{1-15}$,Cys$^{3-11}$]pET, [Cys$^{1-11}$,Cys$^{3-15}$]pET [Cys(Acm^{3,11})]pET and [Cys(Acm^{1,15})]pET as indicated by arrows. Each panel shows a typical trace from the same cell preparations. Calculated values for $[Ca^{2+}]_i$ are shown on the ordinates. One-min interval is underlined.

potent as pET in increasing $[Ca^{2+}]_i$ with both initial transient and subsequent sustained phases (Fig. 3a). Neither pET(1-20), pET(1-15), nor pET(16-21) affected $[Ca^{2+}]_i$ response, although pET(1-21) was still capable of increasing $[Ca^{2+}]_i$ in these pre-treated cells (Fig. 3b). [Cys^{1-15},Cys^{3-11}]pET had greater effect on stimulating $[Ca^{2+}]_i$ increase than [Cys^{1-11},Cys^{3-15}]pET, whereas [$Cys(Acm)^{3,11}$]- and [$Cys(Acm)^{1,15}$]-pET were ineffective (Fig. 3c).

DISCUSSION

Using a variety of synthetic analogs, the present study clearly demonstrates the structure/activity relationship of a novel endothelium-derived vasoconstrictor pET. The activities of pET and related peptides in receptor binding almost correlated with those of $[Ca^{2+}]_i$ response in rat VSMC, suggesting that increase in $[Ca^{2+}]_i$ induced by pET is receptor-mediated.

From the present results, truncated analogs, pET(1-15) and pET(16-21), failed to affect receptor binding and $[Ca^{2+}]_i$ response. Surprisingly, removal of even a single residue at the C-terminus (Trp^{21}) resulted in a complete loss of receptor binding activity and subsequent $[Ca^{2+}]_i$ response. These data are compatible with those of Kimura et al. (8) and ours (5) in which pET(1-15) and pET(16-21) were inactive and pET(1-20) was three orders of magnitude less active than pET(1-21) in constricting porcine coronary artery and rat pulmonary artery. Taken together, these data indicate that the C-terminal Trp^{21} residue plays an essential role in interacting with ET receptor, stimulating $[Ca^{2+}]_i$ increase, and subsequent vasoconstriction. While oxidation of Met^{7} residue, although slightly decreased binding affinity, had stimulatory effect on $[Ca^{2+}]_i$ similar to that of intact pET, N-terminal extension of two basic amino-acid residues (Lys-Arg) resulted in a marked decrease in receptor

binding activity as well as $[Ca^{2+}]_i$ response. Our results are comparable to those of their vasoconstrictive activities (5). One might speculate that N-terminal elongation may somehow confer steric hindrance on the active sites and/or conformational changes of the ET molecule to interact with its receptor.

Opening of any disulfate bonds at Cys(3,11) or Cys(1,15) by Acm led to a complete loss of receptor binding activity and $[Ca^{2+}]_i$ response. These data are consistent with our recent observation that each of monocyclic pET analogs had no vasoconstrictive activity (5). Therefore, two intramolecular ring structures are important for receptor binding and vasoconstriction.

The present study further shows that $[Cys^{1-15},Cys^{3-11}]$pET is far more potent than $[Cys^{1-11},Cys^{3-15}]$pET in receptor binding and $[Ca^{2+}]_i$ response, which is consistent with their vasoconstrictive activities (5). Two disulfide bonds in the natural product of pET are situated at the positions of Cys(1-15) and Cys(3-11) (1), of which location has recently been shown to be identical to that of snake venom sarafotoxins with high sequence homology to pET (9). In contrast, bee venom apamin, although structurally homologous to pET, has two disulfide bonds at the positions of Cys(1-11) and Cys(3-15) (10). We have shown that apamin has no effect on ^{125}I-pET binding (4), $[Ca^{2+}]_i$ response nor vasoconstriction (unpublished observations). Furthermore, it has recently been shown that sarafotoxin is a potent vasoconstrictor (5,11). Taken collectively, it is strongly suggested that the proper double cyclic structure formed by two intramolecular disulfide linkages between Cys(1-15) and Cys(3-11) is critical for receptor binding to induce vasoconstriction.

It is thus concluded that the C-terminal Trp^{21} residue and the proper double cyclic structure of the pET molecule are essential for receptor binding and $[Ca^{2+}]_i$ increase in rat VSMC.

ACKNOWLEDGMENTS

We thank Drs. M. Yanagisawa and T. Masaki, Institute of Basic Medical Sciences, University of Tsukuba, for helpful discussion. 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, 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. Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. and Masaki, T. (1988) FEBS Lett. 231, 440-444.
3. Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S., Nakajima, K., Watanabe, T.X., Sakakibara, S., Goto, K. and Masaki, T. (1988) Proc. Natl. Acad. Sci. USA, 85, 6964-6967.
4. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagaye, S., Nakajima, K. and Sakakibara, S. (1988) Biochem. Biophys. Res. Commun. 154, 868-875.
5. Kumagaye, S., Nakajima, K., Nishio, H., Kuroda, H., Watanabe, T.X., Kimura, T., Masaki, T. and Sakakibara, S. (1988) In. Peptide Chemistry (ed. M.Ueki), Protein Research Foundation, Osaka (in press).
6. Hirata, Y., Tomita, M., Yoshimi, H. and Ikeda, M. (1984) Biochem. Biophys. Res. Commun. 125, 562-568.
7. Grykiewicz, G., Poenie, M. and Tsien, M. P. (1985) J. Biol. Chem. 260, 3440-3450.
8. Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1988) Biochem. Biophys. Res. Commun. (1988) 156, 1181-1186.
9. Takasaki, C., Tamiya, N., Bdolah, A., Wollberg, Z. and Kochva, E. (1988) Toxicon 26, 543-548.
10. Callewaert, G.L., Shipolini, R. and Vernon, C.A. (1968) FEBS Lett. 1, 111-113.
11. Kloog, Y., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. and Bdolah, A. (1988) Science 242, 268-270.