18. Weinberg, S. *Gravitation and Cosmology* (Wiley, New York, 1972).
19. Searle, L., Sargent, W. L. W. & Bagnuolo, W. G. *Astrophys. J.* **179,** 427–438 (1973).
20. Trimble, V. *Rev. Mod. Phys.* **54,** 1183–1224 (1982).
21. Woosley, S. E. & Weaver, T. A. *A. Rev. Astr. Astrophys.* **24,** 205–254 (1986).
22. Ciardullo, R., Ford, H. C., Neill, J. D., Jacoby, G. H. & Shafter, A. W. *Astrophys. J.* **318,** 520–530 (1987).
23. Tammann, G. in *Supernovae: A Survey of Current Research* (eds Rees, M. J. & Stoneham, R. J.) 371–404 (Reidel, Dordrecht, 1982).
24. Lyne, A. G., Manchester, R. N. & Taylor, J. H. *Mon. Not. R. astr. Soc.* **213,** 613–639 (1985).
25. Narayan, R. *Astrophys. J.* **319,** 162–179 (1987).
26. Clark, D. H. & Caswell, J. L. *Mon. Not. R. astr. Soc.* **174,** 267–305 (1976).
27. Heiles, C. *Astrophys. J.* **315,** 555–566 (1987).
28. Van den Bergh, S., McClure, R. D. & Evans, R. *Astrophys. J.* **323,** 44–53 (1987).
29. Weissman, P. R. in *The Comet Nucleus Sample Return Mission* ESA SP-249 (1986).
30. Klinger, J. in *Ices in the Solar System* (eds Klinger, J. *et al.*) 407–415 (Reidel, Dordrecht, 1985).
31. Bar-Nun, A., Herman, G., Laufer, D. & Rappaport, M. L. *Icarus* **63,** 317–332 (1985).
32. Yamamoto, T., Nakagawa, N. & Fukui, Y. *Astr. Astrophys.* **122,** 171–176 (1983).
33. Stern, S. A. *Icarus* **68,** 276–283 (1986).
34. Delsemme, A. H. in *Comets* (ed. Wilkening, L.) 659–664 (Univ. of Arizona Press, Tucson, 1982).
35. Ney, E. P. in *Comets* (ed. Wilkening, L.) 323–340 (Univ. of Arizona Press, Tucson, 1982).
36. Spitzer, L. *Physical Processes in the Interstellar Medium* (Wiley, New York, 1978).
37. Dwek, E. & Scalo, J. M. *Astrophys. J.* **239,** 193–211 (1980).
38. Seab, C. G. & Shull, J. M. in *Interrelationships among Circumstellar, Interstellar, and Interplanetary Dust* (eds Nuth, J. A. & Stencel, R. E.) NASA CP-2403, 37–54 (1985).
39. deBoer, K. S., Jura, M. & Shull, J. M. in *Exploring the Universe with the IUE Satellite* (ed. Kondo, Y.) 485–516 (Reidel, Dordrecht, 1987).
40. Conti, P. S. & Underhill, A. *O, Of, and Wolf-Rayet Stars* NASA Special Publ. (in press).
41. Yamamoto, T. in *Ices in the Solar System* (eds Klinger, J. *et al.*) 205–219 (Reidel, Dordrecht, 1985).
42. Greenberg, J. M. & d'Hendecourt, L. B. in *Ices in the Solar System* (eds Klinger, J. *et al.*) 185–204 (Reidel, Dordrecht, 1985).

A novel potent vasoconstrictor peptide produced by vascular endothelial cells

Masashi Yanagisawa, Hiroki Kurihara‡, Sadao Kimura, Yoko Tomobe, Mieko Kobayashi*, Youji Mitsui*, Yoshio Yazaki†, Katsutoshi Goto & Tomoh Masaki§

Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan
* Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Ibaraki 305, Japan
† Third Department of Internal Medicine, University of Tokyo, Hongo, Tokyo 113, Japan

An endothelium-derived 21-residue vasoconstrictor peptide, endothelin, has been isolated, and shown to be one of the most potent vasoconstrictors known. Cloning and sequencing of preproendothelin complementary DNA shows that mature endothelin is generated through an unusual proteolytic processing, and regional homologies to a group of neurotoxins suggest that endothelin is an endogenous modulator of voltage-dependent ion channels. Expression of the endothelin gene is regulated by several vasoactive agents, indicating the existence of a novel cardiovascular control system.

FOLLOWING the discovery in 1980 of endothelium-dependent vasodilation by Furchgott and Zawadzki[1], vascular endothelium has been recognized as an important functional unit involved in the regulation of vascular smooth muscle tonus. It has been hypothesized that, when stimulated by vasoactive agents such as acetylcholine and bradykinin, endothelial cells (EC) secrete short-lived endothelium-derived relaxing factor(s) (EDRF), causing relaxation of underlying smooth muscle cells (see refs 2 and 3 for reviews). One EDRF has recently been identified as nitric oxide or a closely related substance[4]. Vasoconstriction dependent on or enhanced by intact endothelium has also been observed in response to various chemical and physical stimuli such as, noradrenaline[5], thrombin[5], hypoxia[6,7], increased transmural pressure[8] and mechanical stretch[9]. Augmentation of noradrenaline-induced vascoconstriction by anoxia[5,10] and neuropeptide Y[11] has also been found to be endothelium-dependent. Diffusible factor(s) which mediate these constrictive reactions have yet to be identified. Recent reports have described a protease-sensitive vasoconstrictor activity in supernatants of cultured EC[12–14]. This activity is dependent on the presence of extracellular Ca^{2+} and is not affected by blocking the action of α-adrenergic, cholinergic, serotonergic or histaminergic neurotransmitters. Production of this peptidergic substance by EC is influenced by several of the chemical factors listed above[14,15].

We have now isolated a potent vasoconstrictor peptide from the culture supernatant of porcine aortic EC, determined its amino-acid sequence, and molecularly cloned the peptide precursor. This peptide, endothelin, does not belong to any previously known peptide family. The endothelin sequence, however, shows local homologies to a certain group of peptide neurotoxins that act on voltage-dependent Na^+ channels, suggesting that endothelin also acts directly on membrane channels. Studies with preproendothelin cDNA reveal that the precursor peptide is proteolytically processed in an unusual way and that its biosynthesis in cultured EC is regulated at the transcriptional level in response to various chemical and mechanical stimuli.

Purification and structure

The supernatant from confluent monolayer cultures of porcine aortic EC causes an endothelium-independent, slow-onset contraction when added to porcine coronary artery strips at a final concentration of 10% v/v or more (data not shown). Unconditioned medium, or medium conditioned with human fibroblast IMR-90 cells, has no effect. Pretreatment of the conditioned medium with 1 $\mu g\,ml^{-1}$ trypsin at 37 °C for 2 h abolishes the endothelin activity. The activity is also found in the serum-free conditioned medium, and no significant decrease of the activity was observed after a long-term (4–5 weeks) serum-free maintenance of the EC culture. These observations indicate that endothelin is not a derivative of a serum component.

That the confluent EC monolayer can be maintained for several weeks even in protein-free culture medium greatly facilitated its purification. We purified the vasoconstrictor from concentrated serum-free conditioned medium, by collecting active fractions after anion-exchange column chromatography and two steps of reversed-phase HPLC (Fig. 1*a–c*). The final endothelin fraction, corresponding to the absorbance peak at the arrow in Fig. 1*c*, was eluted as a single peak on analytical anion-exchange and reversed-phase HPLCs. In one series of experiments, 2.9 nmol purified endothelin was obtained from 9.6 l of the medium conditioned for 5 days with approximately 4×10^9 cells. The amino-acid composition, determined by acid hydrolysis, was: Asx, 2.05; Ser, 2.70; Glx, 1.08; Cys, 1.37, (as cystine); Val,

‡ Present address: Third Department of Internal Medicine, University of Tokyo, Hongo, Tokyo 113, Japan.
§ To whom correspondence should be addressed.

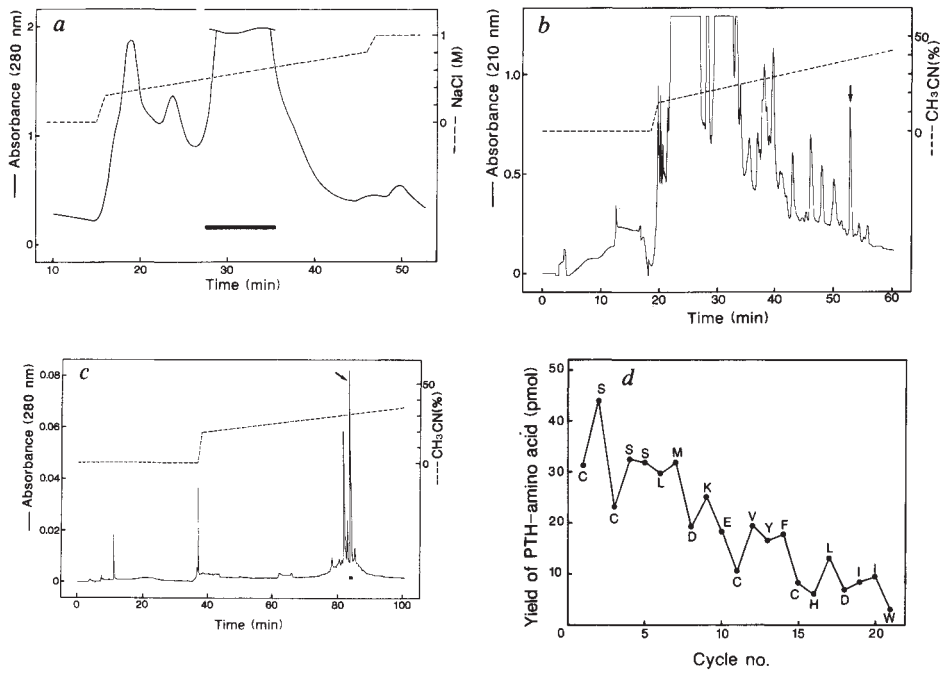


Fig. 1 Purification and sequence analysis of porcine endothelin. *a*, Anion-exchange chromatogram of concentrated serum-free EC-conditioned medium. Concentrated medium (20 ml) was directly loaded onto a DEAE-Toyopearlpak 650 M column (2.2 × 20 cm; Tosoh) connected to a Beckman model 342 HPLC system and equilibrated with 20 mM Tris-HCl (*p*H 7.0), and a linear gradient of NaCl (broken lines) was applied at a flow rate of 4 ml min^{-1}. The eluate absorbance at 210 and 280 nm was recorded and the active fraction designated by a solid bar was collected. *b*, Reverse-phase HPLC. The active fraction from the anion-exchange chromatography was directly applied on a Unisil-Q C_{18} column (7.6 × 250 mm; Gasukuro-Kogyo) equilibrated with 0.1% trifluoroacetic acid (TFA). A gradient of acetonitrile was used at a flow rate of 3 ml min^{-1}. Endothelin activity was co-eluted with the absorbance peak designated with an arrow. *c*, Second reverse-phase HPLC. The active fraction in *b* was further purified on a Chemcosorb 5-ODS-H column (4.6 × 250 mm; Chemco), with a gradient of acetonitrile at a flow rate of 1 ml min^{-1} in the presence of 0.1% TFA. Arrow, final endothelin fraction. *d*, Peptide sequence analysis. The yield of phenylthiohydantoin (PTH)-amino acids at each cycle of Edman degradation is shown with the one-letter amino acid notation.

Methods Endothelial cells (EC) were isolated from adult porcine thoracic aortas[37] and grown to a confluent monolayer in Eagle's minimum essential medium (MEM), containing 10% horse serum at 37 °C in 5% CO_2/95% air. Cells were identified by typical 'cobble-stone' morphology and by immunofluorescence to factor VIII antigen. Smooth-muscle cell contamination was less than 0.1%. After passing 10–15 times, cells were grown to confluence in a multi-layered tissue culture flask on a surface area of 12,000 cm^2 (Cellfactory, Nunc). Cells were washed twice with phosphate buffered saline and fed with 3.2 l of serum-free MEM. Medium was changed every 5 days and the conditioned medium was pooled and stored at −20 °C. Pooled conditioned medium was centrifuged at 1,000*g* for 20 min, the supernatant loaded on a 3 × 18 cm C_{18} reverse-phase column (SP-C-ODS, Chemco) equilibrated with 0.1% TFA, and adsorbed material eluted with 30 ml 0.1% TFA/70% acetonitrile. Eluate was extracted twice with 200 ml diethylether and the aqueous phase adjusted to *p*H 7.0 with Tris base. For bioassay of endothelin activity, dilutions of EC-conditioned medium or the HPLC fractions were added directly into the muscle chamber (see Fig. 2 legend). For peptide sequencing ~100 pmol purified endothelin was reduced in a solution of 0.2 M *N*-methylmorpholine acetate (*p*H 8.0), 20 mM 2-mercaptoethanol at 22 °C for 30 min. The reaction was incubated at 22 °C for a further 90 min after adding 4-vinylpyridine to 40 mM. The pyridylethylated endothelin was purified on a C_{18} reverse-phase HPLC and applied to a gas-phase protein sequencer (Model 470A/120A, Applied Biosystems).

0.91; Met, 0.90; Ile, 1.03; Leu, 1.97; Tyr, 0.64; Phe, 1.11; His, 0.99; Lys, 1.00; Trp, 0.98; Thr, Pro, Gly, Ala and Arg, undetected. Furthermore, automated gas-phase peptide sequencing (Fig. 1*d*), and carboxyterminal analysis by hydrazinolysis (data not shown) together showed that porcine endothelin is of relative molecular mass (M_r) 2,492, comprised of 21 amino acid residues with free amino- and carboxy-termini. The four cysteine residues of endothelin were found to form two intrachain disulphide bonds (Fig. 5). Synthetic endothelin was prepared by liquid-phase chemistry, crosslinking the four cysteine residues according to the analytically determined structure. The synthetic endothelin showed complete biological activity, and retention times identical to those of the natural peptide on a C_{18} reverse-phase HPLC and an anion-exchange HPLC (data not shown). Technical details for the determination of the primary structure and the disulphide-bond topology, together with the liquid-phase synthesis of endothelin will be described elsewhere.

Vasoconstrictor/pressor activities

A typical example and the dose-response relationship of the vasoconstrictor effect of endothelin on porcine right coronary artery strips is shown in Fig. 2. Similar results were obtained with strips of rat aortas, cat basilar arteries, rabbit mesenteric arteries, dog mesenteric, femoral and renal arteries, and human mesenteric and pulmonary artery branches (data not shown). The maximum tensions are comparable to those of KCl-induced contraction. The estimated concentration at which endothelin was 50% effective (EC_{50}) in this assay was $4.0 \pm 2.2 \times 10^{-10}$ M. This figure is at least one order of magnitude lower than the reported values for angiotensin II[16], vasopressin[17] or neuropeptide Y[18], indicating that endothelin is the most potent mammalian vasoconstrictor peptide known to date. *In vivo*, intravenous bolus injection of endothelin causes a markedly sustained rise in arterial pressure in anaesthetized, chemically denervated rats (Fig. 2*c*). The pressor effect is more prominent in diastole. Typically, more than 40–60 min is required for return of arterial pressure to the base-line levels.

The endothelin-induced contraction of porcine coronary artery strips is long-lasting and characteristically difficult to wash out, although completely reversed by the addition of 2×10^{-7} M isoproterenol, or 10^{-6} M glyceryl trinitrate (Fig. 2*a*). The constrictive response is resistant to the following antagonists: α-adrenergic (phentolamine, 10^{-6} M), H_1-histaminergic (diphenhydramine, 10^{-6} M), serotonergic (methysergide, 10^{-6} M), cyclooxygenase (indomethacin, 10^{-5} M), and lipoxygenase (nordihydroguiaretic acid, 10^{-4} M). This suggests that endothelin acts directly on the smooth muscle cells. The endothelin-induced contraction is completely inhibited when bathing solution was substituted with Ca^{2+}-free Krebs–Ringer solution containing 1 mM EGTA immediately before the addition of 10^{-9} M endothelin. Furthermore, the vasoconstriction was markedly attenuated in the presence of the Ca^{2+}-channel blocker, nicardipine (10^{-8} M). These findings agree with previous observations[12,13] and suggest that influx of extracellular Ca^{2+} is required for the action of endothelin.

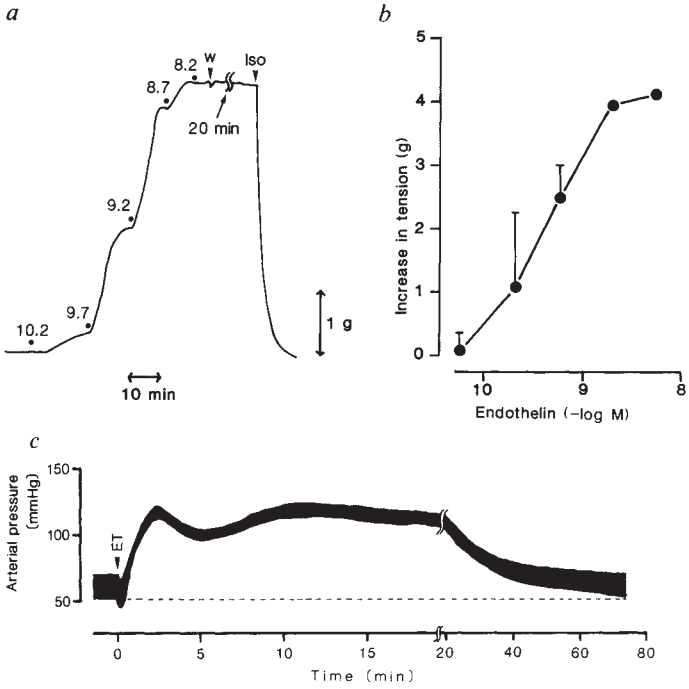
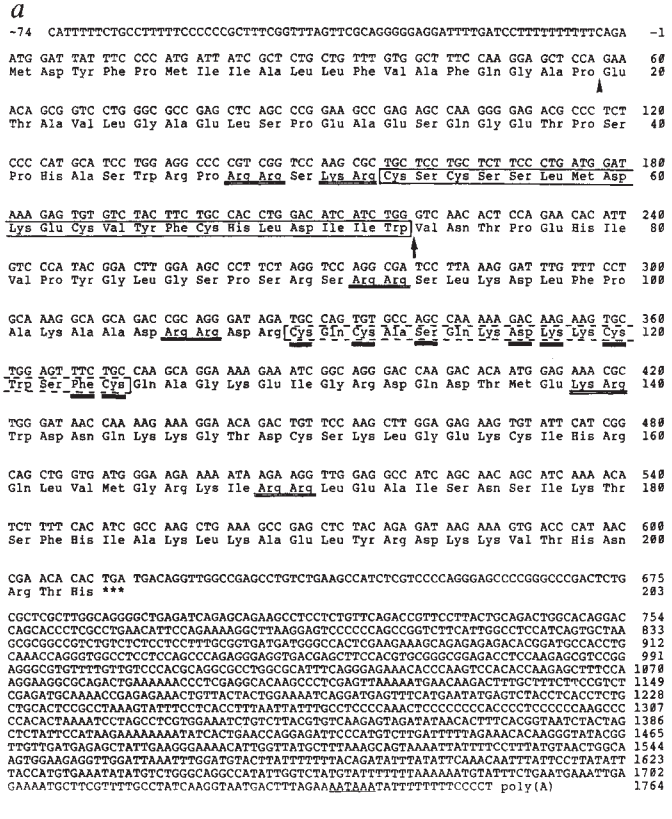


Fig. 2 *a*, Typical constrictive response of porcine right coronary artery strips to cumulatively applied porcine endothelin. Purified endothelin (quantified by amino-acid analysis) was added to the muscle chamber to give the final concentrations shown (designated in terms of negative log molar). W, wash with Krebs–Ringer solution; Iso, 2×10^{-7} M isoproterenol. *b*, Dose–response relationship of the vasoconstrictor effect ($n=4$). Similar results were obtained with synthetic endothelin. *c*, *In vivo* pressor effect of porcine endothelin in an anaesthetized, chemically denervated rat. One nmol kg^{-1} of synthetic endothelin (ET) was applied in bolus intravenously at time 0. Note that the chart speed was changed at 20 min. The base-line diastolic pressure is shown by a broken line. The biphasic response seen in this example was not always observed.

Methods. Right proximal coronary arteries were isolated from fresh adult porcine hearts, brought from a local slaughterhouse within 20 min after death in ice-cold Krebs–Ringer solution (113 mM NaCl, 4.8 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.5 mM glucose). Arterial segments were cut into 2×15 mm helical strips with the intima denuded by rubbing with a small swab, and suspended in 3-ml siliconized glass organ chambers filled with Krebs–Ringer solution maintained at 37 °C and gassed with 95% O_2/5% CO_2. Arterial strips were equilibrated at a passive tension of 1.5 g for two hours. The contraction was measured as an increase in isometric tension with force-displacement transducers (model WT-611T, Nihon Koden). The effectiveness of intimal denudation was assessed by demonstrating that the vasodilatory response to 10^{-9} M Substance P was abolished[3]. For the *in vivo* experiment, a 10-week-old male Wistar rat (350 g body weight) was anaesthetized with urethane (1 g kg^{-1} i.p.), and pretreated with atropine (0.25 mg kg^{-1} i.v.), propranolol (1 mg kg^{-1} i.v.) and bunazosin (1 mg kg^{-1} i.v.). The blood pressure was directly recorded from the right carotid artery with a disposable pressure transducer (model SCK-590, Gould).

Structure of preproendothelin

Screening of $\sim2\times10^6$ clones from a λgt10 cDNA library constructed for porcine aortic EC mRNA with the synthetic DNA probe, described in Fig. 3 legend, resulted in the identification of 38 hybridization-positive clones. Four of these clones, λpET2, λpET4, λpET5 and λpET11 were subjected to further characterization. Genomic Southern analysis probed with the 490 base-pair (bp) *Sac*I fragment of λpET4 insert showed that porcine endothelin is encoded in a single-copy gene (data not shown). The nucleotide sequence of the cDNA inserts is shown in Fig. 3*a*. The 5′-proximal ATG triplet is followed by the only



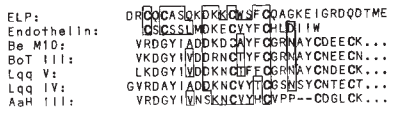


Fig. 3 *a*, Complete nucleotide sequence of porcine preproendothelin cDNA and deduced amino-acid sequence. Arrowhead, putative signal sequence cleavage site predicted by the algorithm described by von Heijne[38]. Paired basic amino-acid residues, Lys–Arg and Arg–Arg, are doubly underlined. Solid lines enclose endothelin sequence; broken lines enclose 'endothelin-like peptide'; solid bars, residues identical to those of endothelin; arrow, the unusual proteolytic processing site at the carboxyl(?)-terminus of endothelin; underline, poly(A) addition signal. *b*, Alignment of endothelin sequence with 'endothelin-like peptide' (ELP) and the amino-terminal regions of several α-scorpion toxins[30]. One-letter amino acid notation. Half-cysteine residues are shown in bold; boxes, identities or conserved amino-acid substitutions[39]. Be M10, *Buthus epeus* neurotoxin M10; BoT III, *Buthus occitanus tunetanus* neurotoxin III; Lqq IV and V, *Leiurus quinquestriatus quinquestriatus* neurotoxin IV and V; AaH III, *Androctonus australis Hector* neurotoxin III.

Methods. RNA was extracted from porcine aortic EC and poly(A) RNA was selected by oligo(dT) cellulose column chromatography[40]. A cDNA library of 2×10^6 independent clones was constructed[41] in λgt10 from 2 μg of the poly(A) RNA. The unamplified library was screened[40] with a single 'optimal' synthetic probe encoding amino-acid residues 7–20 of endothelin. Oligonucleotides ATGGACAAGGAGTGTGTCTAC-TTCTG and GATGATGTCCAGATGGCAGAAGTAGA were synthesized according to the mammalian codon usage statistics[42] with Applied Biosystems model 380A DNA synthesizer, annealed to each other, and labelled[43] to a specific activity of 10^9 c.p.m. μg^{-1} with Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [α-^{32}P]dCTP (3,000 Ci $mmol^{-1}$, Amersham). Hybridization was carried out at 42 °C in 20% formamide. Membranes were washed in $0.2\times$SSC at 30 °C. The restriction fragments of cDNA inserts of four of the hybridization-positive phages, λpET2, λpET4, λpET5 and λpET11, were subcloned into plasmid pUC118 or pUC119. The extent of the four cDNA inserts were: λpET2, nucleotide −74 to 501; λpET4, −2 to poly(A); λpET5, 62 to poly(A); λpET11, −13 to poly(A). The recombinant plasmids were rescued as single-stranded DNA and sequenced by the dideoxy-chain-termination method[44]. The complete sequences of the four inserts were independently determined, both strands being completely covered in the translated regions.

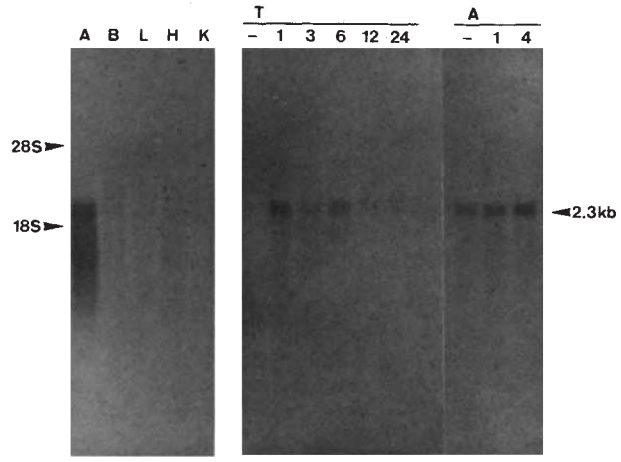


Fig. 4 Northern analysis of preproendothelin mRNA from various porcine tissues (left) and cultured porcine aortic EC exposed to thrombin or the Ca^{2+} ionophore A23187 (right). Tissue RNA (20 μg per lane) was extracted by the LiCl-urea method[45] from adult porcine aortic intima (A), whole brain (B), lung (L), right atrium (H), and kidney (K). As many macroscopic blood vessels as possible were removed from the tissues.

Intimal tissue was scraped off from fresh porcine thoracic aortas directly into the LiCl-Urea lysis solution. RNA was electrophoresed through a 1.2% agarose/formaldehyde gels, transferred to GeneScreen Plus membranes (NEN) and hybridized as recommended by the manufacturer with the insert of λpET4 labelled using [α-^{32}P]dCTP (3,000 Ci mmol^{-1}, Amersham) to a specific activity of 8×10^8 c.p.m. μg^{-1} by the random-primed labelling method[46]. The autoradiograph was exposed for 7 days. RNA from confluent EC (5 μg per lane) was extracted immediately before (–), and at the designated number of hours after, the addition of 2 NIH U ml^{-1} bovine thrombin (T) or 10^{-6}M A23187 (A). Autoradiographs were exposed for 10 h. After autoradiography, the membranes were rehybridized with a myosin regulatory light-chain cDNA pGMRL7E1 (our unpublished work) for an internal standard of total mRNA content; all lanes in each series of experiments showed similar signal intensities. Signal intensities cannot be directly compared between the thrombin series and the A23187 series as different stocks of EC were used.

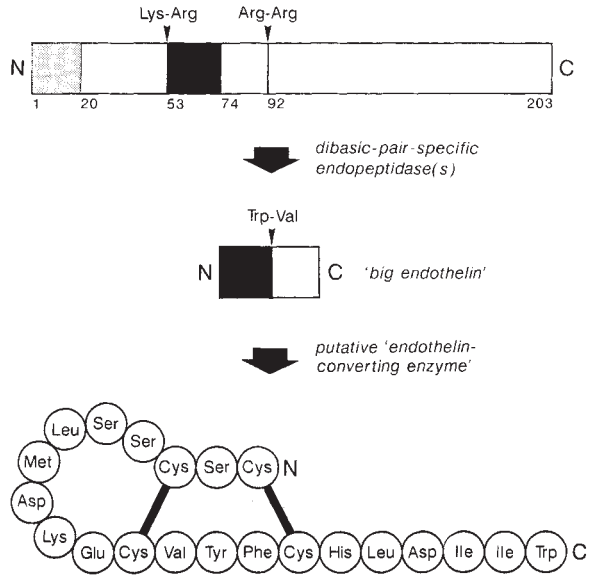


Fig. 5 Possible pathway of endothelin biosynthesis. The putative secretory signal sequence and the endothelin sequence are shown by the shaded and closed boxes, respectively. The rest of the proendothelin sequence is indicated by an open box. A 39-amino-acid residue intermediate, or 'big endothelin', is considered to be generated from proendothelin by the proteolytic cleavages at paired basic residues. Mature endothelin is then produced through an unusual, previously unknown processing by a putative 'endothelin converting enzyme'.

long open-reading frame, which includes the endothelin sequence. The sequence around this ATG fits the consensus sequence for eukaryotic translation initiation sites[19] and the first 19 residues of the deduced amino-acid sequence are characteristic of a secretory signal sequence, that is a hydrophobic core followed by residues with small polar side-chains[20]. The 203-residue preproendothelin sequence deduced from this long open-reading frame is shown under the nucleotide sequence in Fig. 3*a*. The existence in vascular endothelium of mRNA encoding the prepro- form of endothelin indicates that endothelin is produced by *de novo* synthesis and processing in a manner similar to that of many peptide hormones and neuropeptides.

As anticipated, paired basic amino-acid residues Lys 51–Arg 52, which are recognized by processing endopeptidases[21], directly precede the endothelin sequence (Fig. 3*a*). Surprisingly, however, no dibasic-pair is found thereafter until Arg 92–Arg 93. This indicates that the mature endothelin found in the EC-conditioned medium is generated via previously unknown, unusual proteolytic processing between Trp 73 and Val 74 presumably involving an endopeptidase with a chymotrypsin-like specificity. Artefactual proteolysis during the peptide purification is unlikely because the vasoconstrictor activity is always exclusively associated with 21-residue endothelin (Fig. 1; 4 separate experiments). This putative 'endothelin-converting enzyme', could be present in one of three forms: (1) a soluble intracellular enzyme; (2) membrane-bound like the angiotensin converting enzyme[22]; or (3), secreted and active outside the cell. A presumptive pathway of the production of mature endothelin is schematically presented in Fig. 5.

Interestingly, the sequence of preproendothelin from Cys 110 to Cys 124 shows a significant homology to the amino-terminal 15 residues of endothelin (Fig. 3*b*). Eight out of 15 residues (53%) are identical and, more significantly, the relative positions of the four cysteine residues are perfectly conserved. Furthermore, this sequence is flanked with the dibasic-pairs Arg 106–Arg 107 and Lys 139–Arg 140. It is possible that this 'endothelin-like peptide' is actually secreted from the EC, and that it possesses some biological activity that was undetectable in the vasoconstriction bioassay used in the purification procedure. Just prior to the stop codon is a potential glycosylation site (Asn-X-Thr/Ser) at residue 200.

Expression of preproendothelin mRNA

Northern blots probed with λpET4 insert showed that a single 2.3 kilobase (kb) preproendothelin mRNA was expressed not only in the cultured EC but also in porcine aortic intima *in situ* (Fig. 4). Preproendothelin mRNA was not detected in porcine brain, atrium, lung or kidney. This implies that little endothelin is produced by the EC of the microvasculature within these tissues and is consistent with the observation that medium conditioned by microvascular EC from human omental fat does not cause significant vasoconstriction[14].

To establish whether the production of endothelin is constitutive or inducible, confluent monolayers of porcine aortic EC were exposed for specified time periods to medium containing various vasoactive agents. Total cellular RNA was extracted from the EC and the amount of preproendothelin mRNA monitored by Northern analysis (Fig. 4). The level of preproendothelin mRNA increased significantly within 1 h of adding thrombin (2 U ml^{-1}) or the Ca^{2+}ionophore A23187 (10^{-6} M). Adrenaline (10^{-6} M) also caused a marked induction of preproendothelin mRNA within 1 h (H.K., *et al.*, manuscript in preparation). These agents are known to cause endothelium-dependent

vasodilation because of the production of EDRF[3] and, in some cases, to cause endothelium-dependent constriction[2,15]. These latter findings are here substantiated by the observed induction of the endothelium-derived constrictor molecule. In addition, preliminary experiments showed that when EC were cultured in the presence of a medium flow and thus placed under a chronic fluid-mechanical 'shear stress', expression of the prepro-endothelin gene is significantly down-regulated (M. Yoshizumi, personal communication). The flow-dependent inhibition of endothelin production could contribute to endothelium-mediated, flow-induced vasodilatory responses[23,24].

Endothelial cells have few secretory granules when examined under the electron microscope[25]. O'Brien *et al*[14] detected little vasoconstrictor activity in freeze-thaw lysates of the EC that had released the peptidergic constrictor activity into the medium. It is therefore unlikely that endothelin is accumulated in granules and released in response to stimuli. The production of endothelin seems to be regulated predominantly at the level of mRNA transcription.

Physiological implications

The endothelin-type of structure with multiple disulphide bonds within a single, relatively short peptide chain is previously unknown among bioactive peptides of mammalian origin. This type of configuration is often found in a certain group of peptide toxins that act on membrane channels, such as apamin from bee venoms[26], conotoxins from the venoms of fish-hunting sea snails[27] and neurotoxins of scorpion venoms[28]. An examination of the National Biomedical Research Foundation database with the algorithm of Lipman and Pearson[29] detected significant regional homologies between endothelin and α-scorpion toxins[30,31] (Fig. 3*b*). The α-scorpion toxins are ~7,500 M_r peptides with four intrachain disulphide bridges. They bind to tetrodotoxin-sensitive Na^+ channels and inhibit the inactivation of the activated channels, thereby blocking neuronal transmission[32]. These findings raise the possibility that endothelin, like these neurotoxins, acts directly on membrane ion channels. The absolute dependency of endothelin-induced vasoconstriction on the presence of extracellular Ca^{2+}, in conjunction with the inhibition of the endothelin-induced contraction by low doses of nicardipine, suggests that the endothelin action is closely associated with the Ca^{2+} influx through the dihydropyridine-sensitive Ca^{2+} channels. The dihydropyridine-sensitive Ca^{2+} channels and the tetrodotoxin-sensitive Na^+ channels have recently been found to belong to the same superfamily of voltage-dependent membrane ion channels[33]. Although further studies are required for the elucidation of the vasoconstriction mechanism, it is tempting to hypothesize that endothelin is an endogenous agonist of the dihydropyridine-sensitive Ca^{2+} channels.

The observations presented in this study suggest that a novel endothelium-mediated regulation exists in the mammalian cardiovascular system. The basal tonus of vascular beds *in vivo* can be influenced by the level of endothelin production, because endothelin mRNA is actively expressed in EC *in situ* (Fig. 4). Our results support the previous findings that EC produces both EDRF and endothelin in response to various chemical and haemodynamical conditions[34,35]. However, the time-course of the induction and the decay of these mutually antagonizing substances could differ considerably. EDRF may be involved in the rapid local control of vascular tonus, whereas endothelin may contribute to a long-term regulation. The nature of *in vivo* endothelin production requires further study, as cultured EC may approximate more closely to a state of 'injury' *in vivo*[36]. Endothelin, having a potent, strong and characteristically long-lasting vasoconstrictor activity, may be important in the control of systemic blood pressure and/or local blood flow: disturbances in the control of endothelin production could contribute to the pathogenesis of hypertension and that of pathological vascular spasm.

We thank Drs Masahiko Fujino, Chieko Kitada, Shumpei Sakaibara and Terutoshi Kimura for chemical synthesis of endothelin, and Mr Roger Haigh for reading the manuscript. Supported in part by research grants from the Ministry of Education, Science and Culture of Japan, and the University of Tsukuba Project Research.

Received 1 December 1987; accepted 29 January 1988

1. Furchgott, R. F. & Zawadzki, J. V. *Nature* **288,** 373-376 (1980).
2. Furchgott, R. F. *Circ. Res.* **53,** 557-573 (1983).
3. Furchgott, R. F. *A. Rev. Pharmac. Toxicol.* **24,** 175-197 (1984).
4. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. *Nature* **327,** 524-526 (1987).
5. DeMey, J. G. & Vanhoutte, P. M. *Circ. Res.* **51,** 439-447 (1982).
6. Rubanyi, G. M. & Vanhoutte, P. M. *J. Physiol., Lond.* **364,** 45-56 (1985).
7. Holden, W. E. & McCall, E. *Expl Lung Res.* **7,** 101-112 (1984).
8. Harder, D. R. *Circ. Res.* **60,** 102-107 (1987).
9. Katusic, Z. S., Shepherd, J. T. & Vanhoutte, P. M. *Am. J. Physiol.* **252,** H671-H673 (1987).
10. DeMey, J. G. & Vanhoutte, P. M. *J. Physiol., Lond.* **335,** 65-74 (1983).
11. Daly, R. N. & Hieble, J. P. *Eur. J. Pharmac.* **138,** 445-446 (1987).
12. Hickey, K. A., Rubanyi, G., Paul, R. J. & Highsmith R. F. *Am. J. Physiol.* **248,** C550-C556 (1985).
13. Gillespie, M. N., Owasoyo, J. O., McMurtry, I. F. & O'Brien, R. F. *J. Pharmac. exp. Ther.* **236,** 339-343 (1986).
14. O'Brien, R. F., Robbins, R. J. & McMurtry, I. F. *J. cell. Physiol.* **132,** 263-270 (1987).
15. Vanhoutte, P. M., Rubanyi, G. M., Miller, V. M. & Houston, D. S. *A. Rev. Physiol.* **48,** 307-320 (1986).
16. Regoli, D., Park, W. K. & Rioux, F. *Pharmac. Rev.* **26,** 69-123 (1974).
17. Altura, B. M. *Am. J. Physiol.* **219,** 222-229 (1970).
18. Edvinsson, L. *Acta physiol. scand.* **125,** 33-41 (1986).
19. Kozak, M. *Nucleic Acids Res.* **12,** 857-872 (1984).
20. Perlman, D. & Halvorson, H. O. *J. molec. Biol.* **167,** 391-409 (1983).
21. Turner, A. J. *Essays Biochem.* **22,** 69-119 (1986).
22. Johnson, A. R. & Erdos, E. G. *J. clin. Invest.* **59,** 684-695 (1977).
23. Holtz, J. *et al. J. cardiovasc. Pharmac.* **6,** 1161-1169 (1984).
24. Rubanyi, G. M., Romero, J. C. & Vanhoutte, P. M. *Am. J. Physiol.* **250,** H1145-H1149 (1986).
25. Johannessen, J. V. *Electron Microscopy in Human Medicine* Vol. 5, 87-154 (McGraw-Hill, New York, 1980).
26. Habermann, E. *Science* **177,** 314-322 (1972).
27. Olivera, B. M. *et al. Science* **230,** 1338-1343 (1985).
28. Fontecilla-Camps, J. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **77,** 6496-6500 (1980).
29. Lipman, D. J. & Pearson, W. R. *Science* **227,** 1435-1441 (1985).
30. Kopeyan, C., Martinez, G. & Rochat, H. *FEBS Lett.* **181,** 211-217 (1985).
31. Jover, E., Couraud, F. & Rochat, H. *Biochem. biophys. Res. Commun.* **95,** 1607-1614 (1980).
32. Catterall, W. A. *A. Rev. Biochem.* **55,** 953-985 (1986).
33. Tanabe, T. *et al. Nature* **328,** 313-318 (1987).
34. Vanhoutte, P. M. & Rimele, T. J. *J. Physiol., Paris* **78,** 681-686 (1983).
35. Vanhoutte, P. M. *Blood Vessels* **24,** 141-144 (1987).
36. Ross, R. *New Engl. J. Med.* **314,** 488-500 (1986).
37. Gajdusek, C. M. & Schwartz, S. M. *In Vitro* **19,** 394-402 (1983).
38. von Heijne, G. *Nucleic Acids Res.* **14,** 4683-4690 (1986).
39. Dayhoff, M. O., Schwartz, R. M. & Orcutt, B. C. in *Atlas of Protein Sequence and Structure* Vol. 5, Suppl. 3 (*ed.* Dayhoff, M. O.) 345-352 (National Biomedical Research Foundation, Silver Spring, Maryland, 1978).
40. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
41. Huynh, T. V., Young, R. A. & Davis, R. W. in *DNA Cloning: A Practical Approach*, Vol. 1 (ed. Glover, D. M.) 49-78 (IRL, Oxford, 1985).
42. Lathe, R. *J. molec. Biol.* **183,** 1-12 (1985).
43. Lauffer, L. *et al. Nature* **318,** 334-338 (1985).
44. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74,** 5463-5467 (1977).
45. Clemens, M. J. in *Transcription and translation: A Practical Approach* (*ed.* Hames, B. D. & Higgins, S. J.) 211-230 (IRL, Oxford, 1984).
46. Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132,** 6-13 (1983).