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# The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes

(cardiovascular control/vasoconstrictor/vascular endothelium/synthetic peptide/DNA cloning)

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ABSTRACT **Three distinct human endothelin-related genes were cloned by screening a genomic DNA library under a low hybridization stringency with a synthetic oligonucleotide probe encoding a portion of the endothelin sequence. Genomic Southern blot analysis with the same oligonucleotide probe showed three corresponding chromosomal loci not only in the human genome but also in porcine and rat genomes. The nucleotide sequences of the three human genes were highly conserved within the regions encoding the 21-residue (mature) endothelins, in spite of the fact that the immediately upstream exon sequences, which encode a part of the propeptides, retained little similarity. Moreover, each of the human genes predicted a putative 21-residue peptide, similar to but distinct from each other: (*i*) the "classical" endothelin (ET-1), (*ii*) [Trp$^6$,Leu$^7$]endothelin (ET-2), and (*iii*) [Thr$^2$,Phe$^4$,Thr$^5$,Tyr$^6$, Lys$^7$,Tyr$^{14}$]endothelin (ET-3). Synthetic ET-1, ET-2, and ET-3 were prepared according to the deduced amino acid sequences, and the biological activities were assayed by contraction of isolated porcine coronary artery strips and by intravenous injection to anesthetized rats. All these synthetic peptides produced strong vasoconstrictor and pressor responses. However, the quantitative profiles of the pharmacological activities were considerably different among the three isopeptides, suggesting the possible existence of endothelin receptor subtypes.**

Endothelin is a potent vasoconstrictor/pressor peptide originally characterized from the culture supernatant of porcine aortic endothelial cells and consists of 21 amino acid residues with two sets of intrachain disulfide linkages (1). Sequence analysis of cloned cDNAs for porcine (1) and human (2) endothelin precursors showed that endothelin is produced in endothelial cells from an ≈200-residue prepropeptide much like many peptide hormones and neuropeptides. A presumptive 39-residue (porcine) or 38-residue (human) "big endothelin" is thought to be generated from the preproendothelin; the 21-residue (mature) endothelin is produced through an unusual proteolytic processing of big endothelin between Trp$^{21}$ and Val$^{22}$ residues. The amino acid sequences of mature porcine and human endothelin are identical. Preproendothelin mRNA has been detected not only in the cultured endothelial cells but also in porcine aortic endothelium *in vivo*, and the mRNA expression is markedly influenced by various chemical and mechanical stimuli to the endothelial cells (1, 10). These observations suggest an important role of endothelin in regulation of the mammalian cardiovascular system.

In addition to the potent vasoconstrictor and pressor actions, endothelin has been reported to produce a wide spectrum of biological effects: regional vasodilatory effects *in vivo* (3); stimulation of proliferation of vascular smooth muscle cells and fibroblasts (4, 9); contraction of airway and intestinal smooth muscles (5, 6); positive inotropic and chronotropic effects on the myocardium (7, 8); release of icosanoids and/or endothelium-derived relaxing factor from vascular beds (6); stimulation of atrial natriuretic peptide secretion from atrial cardiocytes (11); inhibition of renin release from the glomerulus (12, 28); and modulation of norepinephrine release from sympathetic terminals (13). Tissue autoradiographic studies with $^{125}$I-labeled endothelin in rats have demonstrated that specific high-affinity binding sites for endothelin are distributed not only in vascular smooth muscles but also widely in various noncardiovascular tissues, including the central nervous system (14). We and others have recently discovered that endothelin and a group of peptide toxins from snake venom, sarafotoxins S6, almost certainly have a common evolutionary origin (15, 16). The existence of cognate peptides of endothelin in the apparently unrelated organ (exocrine venomous glands) of the snake suggests that the "classical" endothelin is not necessarily the only mammalian version of this peptide family. Actually, multiple isoforms of biologically active peptides encoded in separate precursor genes are often found in a single mammalian species—e.g., endogenous opioids (17), vasopressin/oxitocin (18), tachykinins (19), calcitonin gene-related peptides (20), and atrial/brain natriuretic peptides (21).

In search of the possible existence of endothelin-related peptide(s) at the DNA level, we have encountered three distinct human genes for putative endothelin precursors. Interestingly, the three genes each encode closely related but different peptides in the endothelin family. Furthermore, the synthetic peptides with the predicted amino acid sequences showed different profiles of pharmacological activities when assayed on isolated arterial smooth muscle strips and on anesthetized rats *in vivo*.†

## MATERIALS AND METHODS

**Cloning and Sequencing of Endothelin-Related Genes.** A human genomic DNA library constructed in λEMBL3 was obtained from Clontech. The library was screened by hybridization with a single synthetic oligonucleotide probe encoding amino acid residues 7–20 of porcine/human endothelin as described (1). Plaque hybridization was performed at 42°C in the presence of 20% formamide, and the membranes were washed in 30 mM NaCl/3 mM sodium citrate, pH 7.0/1% $NaDodSO_4$ at 30°C. The hybridization-positive clones were plaque purified and the relevant restriction fragments of the inserts were subcloned in pUC118/119 plasmids. The plasmids

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04522).

were rescued as single-stranded DNA and sequenced from both strands by the dideoxynucleotide chain-termination method (22). Other standard recombinant DNA procedures were carried out as described (23).

**Synthetic Peptides.** The three predicted peptides from the cloned human genomic DNA—i.e., ET-1, ET-2, and ET-3—were each assembled by using an Applied Biosystems model 430A peptide synthesizer and were purified as described (24, 25). The homogeneity of the final products was confirmed by analytical reverse-phase HPLC and amino acid analysis. Peptides were dissolved to 100 μM in 150 mM NaCl/10 mM sodium phosphate, pH 7.2, containing 0.05% bovine serum albumin (Sigma, Fraction V), and stored in aliquots at −20°C until use. The peptide stock solution was serially diluted with the same buffer immediately before use.

**Assay of Biological Activities.** *In vitro* vasoconstrictor activity of the synthetic peptides was assayed by measuring isometric contractions of de-endothelialized helical strips of porcine coronary artery suspended in a Krebs–Ringer solution as described (1, 24). To avoid nonspecific adsorption of the peptides, all glass containers and organ baths were thoroughly siliconized with dimethyldichlorosilane. For the assay of *in vivo* pressor activity, male Wistar rats weighing 300–350 g were anesthetized with urethane (1 g/kg i.p.) and pretreated with atropine (0.25 mg/kg i.v.), propranolol (1 mg/kg i.v.), and bunazocine (1 mg/kg i.v.). Additional maintenance doses (one-half of the initial doses) of the autonomic blockades were administered every 3–4 hr. Each animal was given only one bolus of a randomly selected peptide. One nanomole of the peptide in 50 μl of buffer was bolus injected from a cannula inserted in the right femoral vein. The blood pressure was recorded from a cannula, which was placed in the right carotid artery and connected to a pressure transducer.

## RESULTS

**Endothelin-Related Loci in Mammalian Genomes.** Southern blot analysis of human, porcine, and rat genomic DNA was performed under low hybridization stringency with a 42-mer synthetic oligonucleotide probe corresponding to amino acid residues 7–20 of endothelin (see Fig. 2). The oligonucleotide probe was designed to minimize possible base mismatches due to the codon redundancy, considering the mammalian codon usage statistics (1) (see Fig. 3). Three different restriction fragments were always detected in these mammalian species irrespective of the restriction endonucleases used. This indicates the existence in mammals of three chromosomal loci that encode endothelin or similar peptides.

**Cloning of Human Endothelin-Related Genes.** Approximately $10^6$ clones from a λEMBL3 human genomic DNA library were screened by plaque hybridization under low stringency with the above described oligonucleotide probe. About 40 hybridization-positive plaques were detected, and the restriction mapping of the cloned phages revealed that all inserts fell into three distinct groups (Fig. 1). Representative clones from each group, λghET-1, λghET-2, and λghET-3, were subjected to further analysis. The restriction maps of these three clones were fully consistent with the results from the human genomic Southern blots (Fig. 2 *Left*), indicating that the inserts of these phages are authentic copies of the genomic loci.

In an attempt to determine the tissue expression of these genes, we performed a Northern blot analysis of mRNA from human tissues obtained from surgical procedures, with the preproendothelin cDNA pHET4-3 (2), the 0.3-kilobase (kb) *Sac* I fragment of λghET-2, and the 0.32-kb *Pst* I fragment of λghET-3 as probes. Tissues examined were frontal cortex, atrium, kidney, liver, spleen, stomach, placenta, testis, ovary, uterus, and cultured umbilical vein endothelial cells. However, we failed to find any detectable amount of hybridizable mRNA in these tissues under a conventional Northern blot analysis, except that expression of the λghET-1 locus was, as expected, observed in the cultured endothelial cells (data not shown).

**Nucleotide and Encoded Amino Acid Sequences.** Partial nucleotide sequences of λghET-1, λghET-2, and λghET-3 are aligned with each other and with the oligonucleotide probe in Fig. 3. The sequences from nucleotides 97–138 matched well with the oligonucleotide probe (37 or 38 of 42 bases). The deduced amino acid sequences for the reading frames corresponding to that of the oligonucleotide probe are also shown under the nucleotide sequences. λghET-1 was found to correspond exactly to the human preproendothelin cDNA pHET4-3 (2), encoding the classical endothelin. Therefore, we concluded that λghET-1 represents the human (classical) preproendothelin gene. In contrast, two other loci encoded a peptide that is highly similar to but distinct from the classical endothelin: λghET-2 for [$Trp^6$,$Leu^7$]endothelin and λghET-3 for [$Thr^2$,$Phe^4$,$Thr^5$,$Tyr^6$,$Lys^7$,$Tyr^{14}$]endothelin. Many of the amino acid variations are substitutions with chemically similar amino acid residues. Most significantly, the positions of the four cysteine residues, which might be an important determinant of the higher structure of the peptides, are perfectly conserved. We hereafter designate the peptides encoded in λghET-1, λghET-2, and λghET-3 as endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), respectively.

We have recently cloned and sequenced an endothelin-related gene in the rat (24). Although the tissue(s) that expresses the gene was unknown, we tentatively called the encoded peptide rat endothelin. Interestingly, however, the rat endothelin is now found to be identical to human ET-3. It seems reasonable to consider that the replacement of six amino acid residues between the rat endothelin and porcine/human endothelin is not due to a species difference, but that rat endothelin is actually the rat counterpart of ET-3, a distinct isoform of endothelin.

The encoded amino acid sequences of ET-1, ET-2, and ET-3 are directly preceded by a conserved pair of basic amino acid residues, (Arg/Lys)-Arg, which is the site of posttranslational proteolytic processing (26). However, no dibasic pair

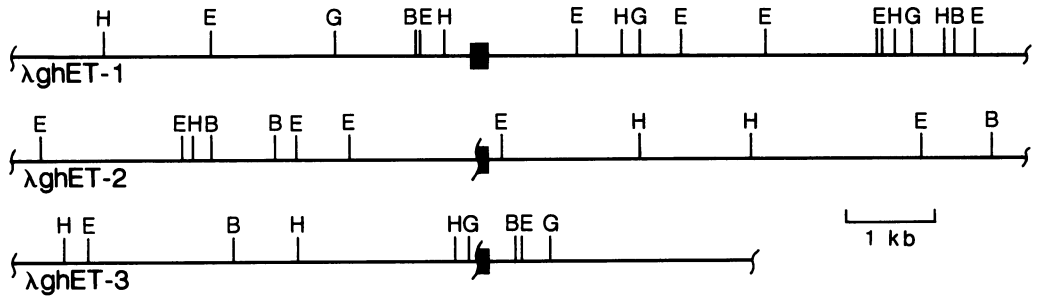


FIG. 1. Partial restriction maps of three human endothelin-related genes cloned in λghET-1, λghET-2, and λghET-3. Presumptive exons encoding mature endothelins are shown as solid boxes. B, *Bam*HI; E, *Eco*RI; G, *Bgl* II; H, *Hin*dIII.

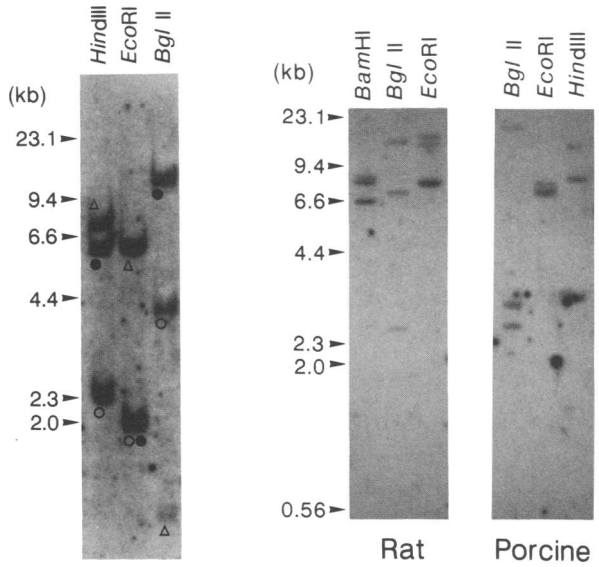


FIG. 2. Southern blots of human (*Left*) and rat and porcine (*Right*) genomic DNA. (*Left*) Hybridization signals corresponding to the loci cloned in λghET-1, λghET-2, and λghET-3 are marked by ○, ●, and △, respectively; note that two 2.0-kb *Eco*RI fragments are detected as a doublet. DNA (10 μg per lane) was digested in completion with the restriction enzymes designated, transferred to GeneScreen*Plus* membranes (DuPont) as recommended by the manufacturer, and hybridized with the labeled synthetic oligonucleotide probe (ref. 1; Fig. 3) at 42°C in 1 M NaCl/20% (vol/vol) formamide/1% $NaDodSO_4$/salmon sperm DNA (250 μg/ml). The membranes were washed several times in 0.3 M NaCl/30 mM sodium citrate, pH 7.0/1% $NaDodSO_4$ at 22°C and in the same solution at 60°C for 10 min and autoradiographed with intensifying screens at −80°C for 4–7 days.

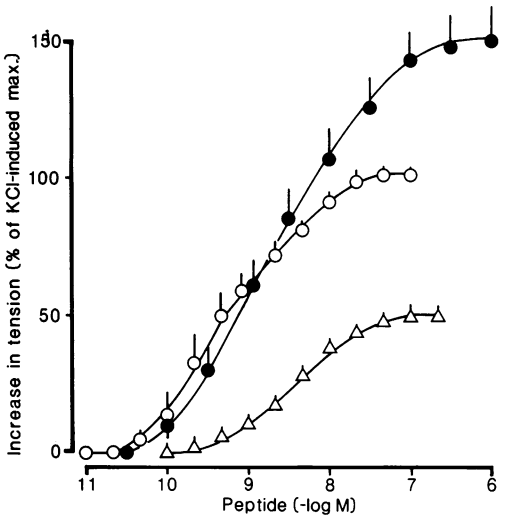


FIG. 4. Dose–response relationships for constrictive responses of de-endothelialized porcine coronary artery strips to cumulatively applied endothelins ($n$ = 6). ○, ET-1; ●, ET-2; △, ET-3.

is found in the carboxyl-terminal region of any of the three peptides. This may indicate that ET-2 and ET-3 are, as in the case of ET-1, produced through an unusual proteolytic processing between Trp-(Val/Ile) by an endothelin-converting enzyme system (1).

An exon/intron junction in λghET-1 was found at nucleotide 156, which is 14 nucleotides 3′ from the codon for the carboxyl-terminal tryptophan of ET-1 (Fig. 3). Interestingly, an intron seems to start exactly at the corresponding positions also in λghET-2 and λghET-3, since the sequences here fit well with the consensus sequence for the 5′ end of an intron, GT(A/G)AGT, and the nucleotide sequences suddenly lose similarity to each other at this location.

**Activities of Synthetic ET-1, ET-2, and ET-3.** According to the predicted amino acid sequences (Fig. 3), we synthesized 21-residue ET-1, ET-2, and ET-3 by a solid-phase chemistry, and we assayed the biological activities of the synthetic peptides on the contractile responses of porcine coronary artery strips *in vitro* and on the pressor responses *in vivo* in anesthetized chemically denervated rats. *In vitro*, the three synthetic peptides, at nanomolar concentrations, all exhibited a strong and long-lasting vasoconstrictor activity characteristic of endothelin (Fig. 4). Fifteen to 20 min was typically required to obtain a steady-state tension for each dose of the individual peptides. The quantitative details of the constrictor effect were significantly different among the isopeptides (Table 1). In terms of molar potency, ET-1 was most potent, whereas the maximum contractile tensions were greatest for ET-2. Among the three peptides, ET-3 was the

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                1                                  30                                  60
λghET-1:  GAG CTC AGC GCG GTG GGT GAG AAC GGC GGG GAG AAA CCC ACT CCC AGT CCA CCC TGG CGG CTC CGC CGG TCC AAG CGC TGC TCC TGC
λghET-2:  A.. GG. CAG ..T .CT .CC ACC CTG .AG CA. CCA GCG T.. T.A T.T CA. G.C .AA G.C ACC .A. .TT ... CTT CGC ..T ... ... ...
λghET-3:  CT. .AG G.T C.A AGC CC. .GA .G. CCT ... C.. G.G .AG G.G G.. GAG GGG G.. CCT GA. .A. .A. ..A ... .G. ... ... A.G ...

                          -20                                 -10                    -1   1
ET-1:     Glu Leu Ser Ala Val Gly Glu Asn Gly Gly Glu Lys Pro Thr Pro Ser Pro Pro Trp Arg Lys Arg Arg Ser Lys Arg Cys Ser Cys
ET-2:     Lys Gly Gln  .  Ala Ala Thr Leu Glu Gln Pro Ala Ser Ser Ser His Ala Gln Gly Thr His Leu  .  Leu Arg  .  .  .  .
ET-3:     Leu Gln Gly Pro Ser Pro Gly Ser Pro  .  Gln Glu Gln Ala Ala Glu Gly Ala Pro Glu His His  .  .  Arg  .  .  Thr  .
                                                                                  Sarafotoxin S6b:  .  .  .

 90                                 120                                 150                                 180
TCG TCC CTG ATG GAT AAA GAG TGT GTC TAC TTC TGC CAC CTG GAC ATC ATT TGG GTC AAC ACT CCC GA  gtaagtctctagagggcattgtaaccctattcatt
AGC ... TG. C.C ..C ..G ... ..C ... ... ... ... ... T.. ... ... ..C ... ..G ... ... ..T ..  ..g..ca..c.tg...at.g.ggg.agggggagc.
.TC A.. TAC .A. ..C ..G ... ... ... ... ... .AT ... ... ... ... ... ... ... A.. ... ... ... ..  .......agcctttt.tgg..aggaa.gtgg.tcc
   Probe:  ... ..C ..G ... ... ... ... ... ... ..T ... ... ... ..C

                          10                                  20
Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp Val Asn Thr Pro
 .   .  Trp Leu  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Phe Thr Tyr Lys  .   .   .   .   .   .  Tyr  .   .   .   .   .   .   .   .  Ile  .   .
Lys Asp Met Thr  .   .   .   .  Leu  .   .   .   .  Gln  .  Val  .   .
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FIG. 3. Partial nucleotide and deduced amino acid sequences of human endothelin-related genes cloned in λghET-1, λghET-2, and λghET-3. In the ET-2 and ET-3 sequences, the nucleotide and amino acid residues identical to those of ET-1 are indicated by dots. The putative introns are shown in lowercase letters. The 42-mer synthetic oligonucleotide probe used for the Southern blot analysis (Fig. 2) and for the screening of a human genomic DNA library is shown under the nucleotide sequences. Presumed mature endothelin sequences are indicated by an open box. A peptide toxin, sarafotoxin S6b, from the venom of the burrowing asp *Atractaspis engaddensis* is also aligned with the deduced sequences of endothelins.

Table 1. *In vitro* constrictor activity of human ET-1, ET-2, and ET-3 on porcine coronary artery strips ($n = 6$)

| | Approximate minimum effective dose, M | $EC_{50}$, M | Maximum tension induced, %* |
|---|---|---|---|
| ET-1 | $3 \times 10^{-11}$ | $5.2 \times 10^{-10}$ | 101 ± 1 |
| ET-2 | $1 \times 10^{-10}$ | $1.9 \times 10^{-9}$† | 150 ± 10† |
| ET-3 | $2 \times 10^{-10}$ | $3.7 \times 10^{-9}$† | 49 ± 2† |

*Expressed as percentages of contractile tension induced by 50 mM KCl.
†Significantly different from the values for ET-1 ($P < 0.05$; one-way analysis of variance and Student's unpaired $t$ test).

least potent in either sense. The time courses for the development of contractile tension were not appreciably different among the three endothelins.

The three peptides also produced strong pressor responses in the anesthetized rats *in vivo*. Typical tracings for the changes of the blood pressure in response to a bolus injection (1 nmol/kg) of one of the peptides are shown in Fig. 5. A transient depressor response lasting 1–2 min always preceded the increase in blood pressure, as described (3, 6, 24). Two distinct phases of pressor effects were typically seen in response to ET-1 and ET-2: the early phase immediately followed the depressor response and dominated during the next 3–10 min, whereas the late pressor effect developed 10–20 min after the injection and lasted >1 hr. The peak increases in blood pressure were not significantly different between the two phases. These two separate phases were not clearly seen in the responses to ET-3. It remains to be established whether different pressor mechanisms operate during the early and late phases of the pressor response. The pressor responses were usually accompanied by an increase in the heart rate in the chemically denervated rats. This may be due to the direct chronotropic action of endothelin on the atrial myocardium (8). The quantitative profiles of the pressor actions are summarized in Table 2. The peak pressor effect was smaller in ET-3 but not significantly different between ET-1 and ET-2. The time required for the return of the blood pressure toward the base-line values was longest in ET-2 and shortest in ET-3. Interestingly, however, the initial depressor effect was significantly more profound in ET-3 than in ET-1.

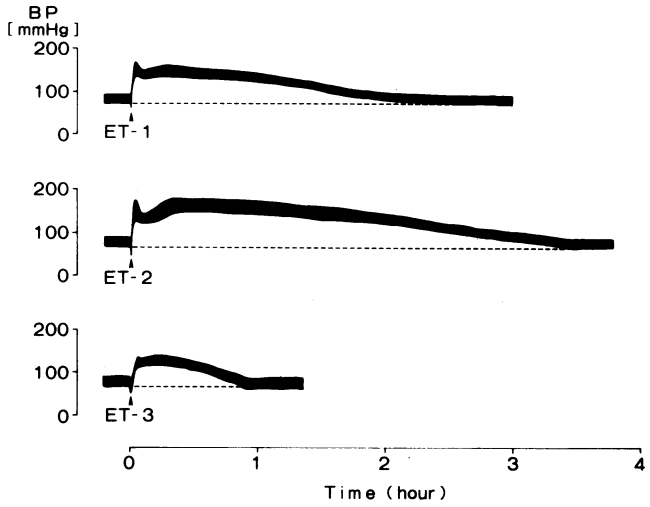


FIG. 5. Typical recordings for pressor responses of anesthetized chemically denervated rats to intravenous bolus injections of ET-1, ET-2, and ET-3 (1 nmol/kg). BP, blood pressure.

## DISCUSSION

The nucleotide sequences encoding amino acid residues 1–25 of the presumptive big forms (1, 2) of the three endothelins (nucleotides 79–153 in Fig. 3) are highly conserved among the three genes, with 77–82% of the nucleotide residues being identical. In contrast, the nucleotide sequences upstream from the encoded dibasic pairs preceding the mature peptides (nucleotides 1–72) as well as the sequences of the putative introns (nucleotides 156–190) are both very poorly conserved. These observations suggest that, although the three genes are evolutionarily relatively distant from each other, the genes evolved from the common ancestral gene under a strong pressure to preserve mature endothelin sequences. The number of endothelin-related genes is demonstrated to be conserved in several mammalian species examined—i.e., human, pig, and rat (Fig. 1). Moreover, one of the three genes in the rat was previously found to encode a peptide identical to ET-3 (24). Although we have not yet been able to demonstrate the transcripts from the genes cloned in λghET-2 and λghET-3 (vascular endothelial cells express ET-1 only), these findings strongly argue against the possibility that these loci are nonfunctional pseudogenes.

In this respect, preliminary studies showed that an ET-3-like immunoreactivity was detected in a homogenate of porcine spinal cord and brain, and this immunoreactivity was coeluted with synthetic ET-3 on a reverse-phase HPLC (S.K., unpublished observation). Thus, ET-3 might be a neural form of endothelin. One possibility is that λghET-2 and/or λghET-3 loci are expressed in a minor cellular subpopulation(s) within specific tissue(s), such as a specific type(s) of neuron in the central nervous system. If these genes are actually functional, more sensitive techniques including specific immunohistochemistry and *in situ* hybridization would elucidate the tissue expression of the genes.

Although all three isopeptides of endothelin are demonstrated in this study to be potent constrictors of arterial smooth muscle *in vitro* and strong pressor agents *in vivo*, the characteristics of the pharmacological activities are quantitatively different among the isopeptides. When assayed on porcine coronary artery strips, the vasoconstrictor activity in terms of maximum tension induced was ET-2 > ET-1 > ET-3. The time required for the recovery of arterial pressure after an intravenous bolus of the peptide in anesthetized rats

Table 2. *In vivo* pressor and depressor activity of human ET-1, ET-2, and ET-3 (1 nmol/kg) in anesthetized chemically denervated rats ($n = 5$)

| | Peak variation of mean blood pressure, mmHg | | | Time required for blood pressure recovery, min | | |
|---|---|---|---|---|---|---|
| | Initial depressor phase | Early pressor phase | Late pressor phase | 50% recovery to base-line levels | 80% recovery to base-line levels | Peak changes in heart rate, $min^{-1}$ |
| ET-1 | −10 ± 2 | 66 ± 5 | 64 ± 3 | 90 ± 5 | 121 ± 7 | 34 ± 11 |
| ET-2 | −16 ± 2 | 68 ± 7 | 72 ± 3 | 119 ± 6* | 159 ± 9* | 18 ± 10 |
| ET-3 | −19 ± 2* | — | 51 ± 4 | 50 ± 11* | 74 ± 15* | 7 ± 4* |

See Fig. 5 and the text for the explanation of the early and late pressor phases; these two phases were not clearly distinguished in ET-3. 1 mmHg = $1.333 \times 10^2$ Pa.
*Significantly different from the values for ET-1 ($P < 0.05$; one-way analysis of variance and Student's unpaired $t$ test).

was also ET-2 > ET-1 > ET-3. Interestingly, these orders of biological potency seem to correlate with the hydrophobicity of the peptides: ET-2 is the most hydrophobic, having one additional tryptophan residue at position 6, whereas ET-3 is the most polar because of the presence of a charged $Lys^7$ residue instead of the hydrophobic $Met^7$ of ET-1. The sequence alignment of the three isopeptides of human endothelin and the recently characterized endothelin-related toxin, sarafotoxin S6b, shows that the sequence variation is more frequent in the amino-terminal regions, especially at residues 4–7. The sequence and hydrophobicity of this variable region might determine the detailed pharmacological characteristics of individual isopeptides. In contrast, in addition to the four cysteine residues at positions 1, 3, 11, and 15, the carboxyl-terminal tail portion including the last tryptophan residue, as well as the cluster of alternating charges, $Asp^8$-$Lys^9$-$Glu^{10}$, is highly conserved among all these peptides. Previous studies on the structure–activity relations of various derivatives of ET-1 have shown that the carboxyl-terminal tryptophan residue and the intact Asp-Lys-Glu sequence are actually important for vasoconstrictor activity (27).

The findings presented in this study suggest the existence of three isoforms of human endothelin, which are distinct from each other in both structure and pharmacological activity. An apparent discordance was found between the *in vivo* initial depressor activities and the vasoconstrictor/pressor activities of the isoforms of endothelin: ET-1 exhibited a more potent constrictor activity than ET-3 both *in vivo* and *in vitro*, whereas ET-3 exerted more profound initial depressor responses *in vivo*. It is tempting to suppose that these peptides from the endothelin family might play differential physiological roles, possibly by interacting with different subtypes of endothelin receptors.

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1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) *Nature (London)* **332,** 411–415.
2. Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. & Masaki, T. (1988) *FEBS Lett.* **231,** 440–444.
3. Wright, C. E. & Fozard, J. R. (1988) *Eur. J. Pharmacol.* **155,** 201–203.
4. Komuro, I., Kurihara, H., Sugiyama, T., Takaku, F. & Yazaki, Y. (1988) *FEBS Lett.* **238,** 249–252.
5. Uchida, Y., Ninomiya, H., Saotome, M., Nomura, A., Ohtsuka, M., Yanagisawa, M., Goto, K., Masaki, T. & Hasegawa, S. (1988) *Eur. J. Pharmacol.* **154,** 227–228.
6. de Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T. D. & Vane, J. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9797–9800.
7. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. & Masaki, T. (1988) *Am. J. Physiol.* **255,** H970–H973.
8. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. & Masaki, T. (1988) *Pflügers Arch.* **413,** 108–110.
9. Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. & Masaki, T. (1989) *J. Biol. Chem.*, in press.
10. Yanagisawa, M., Inoue, A., Takuwa, Y., Mitsui, Y., Kobayashi, M. & Masaki, T. (1989) *J. Cardiovasc. Pharmacol.*, in press.
11. Fukuda, Y., Hirata, Y., Yoshimi, H., Takatsugu, K., Kobayashi, Y., Yanagisawa, M. & Masaki, T. (1988) *Biochem. Biophys. Res. Commun.* **155,** 167–172.
12. Rakugi, H., Nakamaru, M., Saito, H., Higaki, J. & Ogihara, T. (1988) *Biochem. Biophys. Res. Commun.* **155,** 1244–1247.
13. Wiklund, N. P., Ohlen, A. & Cederqvist, B. (1988) *Acta Physiol. Scand.* **134,** 311–312.
14. Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M. & Masaki, T. (1989) *Am. J. Physiol.*, in press.
15. Takasaki, C., Yanagisawa, M., Kimura, S., Goto, K. & Masaki, T. (1988) *Nature (London)* **335,** 303.
16. Kloog, Y., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. & Bdolah, A. (1988) *Science* **242,** 268–270.
17. Douglass, J., Civelli, O. & Herbert, E. (1984) *Annu. Rev. Biochem.* **53,** 665–715.
18. Ruppert, S., Scherer, G. & Schutz, G. (1984) *Nature (London)* **308,** 554–557.
19. Kotani, H., Hoshimaru, M., Nawa, H. & Nakanishi, S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7074–7078.
20. Amara, S. G., Arriza, J. L., Leff, S. E., Swanson, L. W., Evans, R. M. & Rosenfeld, M. G. (1985) *Science* **229,** 1094–1097.
21. Sudoh, T., Kangawa, K., Minamino, N. & Matsuo, H. (1988) *Nature (London)* **332,** 78–81.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
23. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
24. Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S., Nakajima, K., Watanabe, T. X., Sakakibara, S., Goto, K. & Masaki, T. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6964–6967.
25. Kumagaye, S., Kuroda, H., Nakajima, K., Watanabe, T. X., Kimura, T., Masaki, T. & Sakakibara, S. (1988) *Int. J. Pept. Protein Res.* **32,** 519–526.
26. Turner, A. J. (1986) *Essays Biochem.* **22,** 69–119.
27. Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. & Masaki, T. (1988) *Biochem. Biophys. Res. Commun.* **156,** 1182–1186.
28. Takagi, M., Matsuoka, H., Atarashi, K. & Yagi, S. (1988) *Biochem. Biophys. Res. Commun.* **157,** 1164–1168.