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Endothelin: A Novel Peptide in the Posterior Pituitary System

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Endothelin (ET), originally characterized as a 21-residue vasoconstrictor peptide from endothelial cells, is present in the porcine spinal cord and may act as a neuropeptide. Endothelin-like immunoreactivity has now been demonstrated by immunohistochemistry in the paraventricular and supraoptic nuclear neurons and their terminals in the posterior pituitary of the pig and the rat. The presence of ET in the porcine hypothalamus was confirmed by reversed-phase high-pressure liquid chromatography and radioimmunoassay. Moreover, in situ hybridization demonstrated ET messenger RNA in porcine paraventricular nuclear neurons. Endothelin-like immunoreactive products in the posterior pituitary of the rat were depleted by water deprivation, suggesting a release of ET under physiological conditions. These findings indicate that ET is synthesized in the posterior pituitary system and may be involved in neurosecretory functions.

ENDOTHELIN (ET) WAS ORIGINALLY characterized as a 21–amino acid vasoconstrictor peptide from endothelial cells, with a molecular weight of 2492 (*1*). The structure of ET is different from previously known bioactive peptides of mammalian origin (*1*). We have demonstrated a depolarizing action of ET on spinal neurons and the presence of ET in the porcine spinal cord (*2*). Autoradiographic studies in brain tissues also reported a nonvascular pattern of distribution of binding sites for ET (*3*). In addition, sarafotoxins, which are toxins from the burrowing asp (*Atractaspis engaddensis*) that have striking structural and functional similarities to ET, were reported to show high-affinity binding and hydrolysis of phosphoinositides in brain (*4*). These data suggest that ET may have an important role in neuronal function as a neuropeptide. However, the precise localization of ET in brain areas has not yet been elucidated. In the present study, we demonstrate a prominent localization of ET in the paraventricular and supraoptic nuclear neurons in the hypothalamus and in the neurohypophysis (posterior pituitary).

An antiserum was developed against [Arg^{14}]ET(15–21) conjugated with keyhole limpet hemocyanin (KLH) with *m*-maleimidobenzoyl-hydroxysuccinimide ester (*5*, *6*). ET(15–21) corresponds to the COOH-terminal region of ET (ETc), which is homologous in the ET family of proteins (*7*, *8*). Using the antiserum to ETc (ETc antiserum), we performed immunohistochemistry on porcine and rat brain and found strong ET-like immunoreactivity in the posterior pituitary systems of both species. The caudal part of the paraventricular nucleus (PVN) of the porcine hypothalamus (frontal section) is shown in Fig. 1A. A number of PVN neurons, mainly localized near the surface of the third ventricle, were densely immunostained for ET. The distribution of these ET-positive neurons in the PVN was similar to that of oxytocin-producing neurons, also reported to be confined adjacent to the third ventricle (*9*). In the supraoptic nucleus (SON), some neurons were strongly immunostained and others were negative in the same region (Fig. 1B) (*10*). In the rat, magnocellular neurons in PVN and SON were immunostained for ET (Fig. 1, C and D, respectively). Numerous dot-like immunoreactive structures were also found in the posterior pituitary (Fig. 1E). By contrast, antiserum that had been absorbed with ET showed negative immunostaining in the same region (Fig. 1F). These immunohistochemical data suggested the existence of ET-related peptides in these neurosecretory systems.

For the chemical identification of ET in the hypothalamus, we applied the peptide extract prepared from the porcine hypothalamic homogenates to a reversed-phase high-pressure liquid chromatography column and determined ET-like immunoreactivity in each fraction by radioimmunoassay (RIA) with ETc antiserum (*11*). There are three distinct ET-related genes encoding different ET-related peptides in the human genome: ET-1, identical to porcine and human mature ET; ET-2, [Trp^6, Leu^7]ET; and ET-3, [Thr^2, Phe^4, Thr^5, Tyr^6, Lys^7, Tyr^{14}]ET (*8*). The ETc structure is predicted to be preserved among these three peptides (*8*). The existence of three distinct ET-related loci has also been shown in the porcine and rat genomic DNAs (*8*). In the porcine hypothalamic extracts, however, the peaks of ET-like immunoreactivity emerged only at the elution time corresponding to that of ET-1 (Fig. 2), indicating that the major component in the porcine hypothalamus was ET-1.

Furthermore, to obtain direct evidence for the production of ET mRNA in PVN neurons, we examined porcine hypothalamus by in situ hybridization with a ^{35}S-labeled ET-1 complementary RNA probe. Neurons in the region of porcine PVN were heavily labeled, demonstrating the expres-

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sion of ET-1 mRNA (Fig. 3). This result indicates that the presence of ET-like immunoreactivity in PVN neurons is due to synthesis of ET in these cells rather than other mechanisms, for example, uptake of ET from external sources.

Because dehydration is a potent stimulus for the release of vasopressin and probably also oxytocin (*12*), we examined whether water deprivation exerted any effect on the amount of ET-like immunoreactive products in the rat posterior pituitary. After 4 days of water deprivation, immunoreactive products in the posterior lobe had largely disappeared (Fig. 4) (*13*). These findings suggest that ET is released from the posterior pituitary during dehydration.

Endothelin could have one of two roles in the posterior pituitary system. The first is that ET released from the nerve terminals in the posterior lobe may locally modulate the release of the classical neurosecretory hormones vasopressin or oxytocin. Endogenous opiate peptides, which are also localized in the posterior pituitary system, inhibit the release of vasopressin and oxytocin (*14*). By contrast, ET increases the plasma level of vasopressin by intravenous infusion in conscious dogs (*15*). These findings suggest that ET in the posterior pituitary system may modulate the release of vasopressin or oxytocin.

In addition, ET released from the posterior pituitary may act as a circulating hormone similar to vasopressin and oxytocin. Indeed, ET has a wide range of actions on the cardiovascular, renal, and endocrine systems; for example, ET exerts a positive inotropic effect on heart (*16*), inhibits renin release from isolated glomeruli (*17*), induces the release of atrial natriuretic peptide (ANP) from atria (*18*), and increases the plasma levels of vasopressin and epinephrine (*15*). Moreover, the concentration of immunoreactive ET-1 in human plasma measured

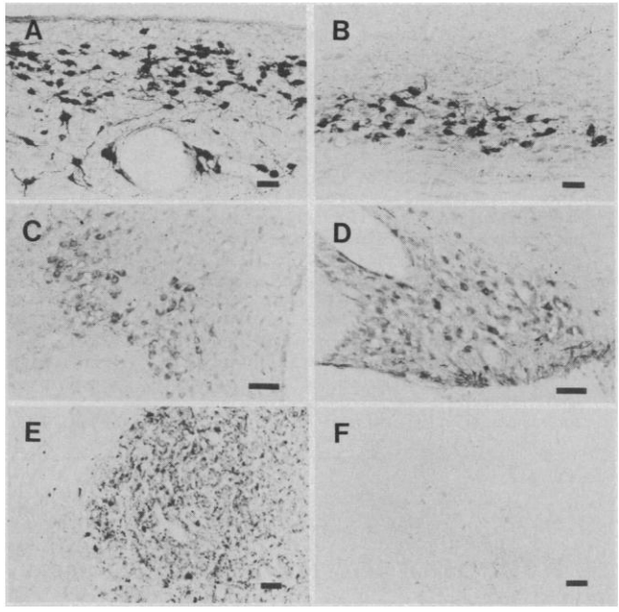

Fig. 1. Immunohistochemical localization of ET-like immunoreactivity in the porcine and rat hypothalamic and posterior pituitary systems with ETc antiserum. (**A**) Porcine PVN; (**B**) porcine SON; (**C**) rat PVN; (**D**) rat SON; (**E**) rat posterior pituitary; and (**F**) rat posterior pituitary stained with absorbed antiserum. Specificity of the immunostaining was examined by the absorption test in which ETc antiserum (1:1000, 1 ml) was neutralized by ET-1 (10 nmol) combined with ET-3 (10 nmol). Absorbed antiserum showed negative immunostaining. A male miniature pig (body weight, 3.5 kg) and male Wistar rats were anesthetized with pentobarbital and perfused intracardially with a 4% paraformaldehyde solution buffered with 0.1*M* phosphate buffer (PB) (*p*H 7.2). Hypothalamic regions and pituitary glands were removed and placed in the same fixative for 2 hours at 4°C. After several rinses in 0.1*M* PB containing 5% sucrose for 24 hours, tissues were immersed in 0.1*M* PB containing 10% sucrose for 2 hours and successively in 0.1*M* PB containing 30% sucrose for 12 hours at 4°C. The hypothalamus was frontally sectioned at 20 μm, and the pituitary gland was transversely sectioned at 10 μm with a cryostat at −20°C. After treatment with 0.3% H_2O_2 in methanol for 30 min, sections were incubated with 2% normal goat serum in 0.01*M* phosphate-buffered 0.15*M* saline (PBS) (*p*H 7.2) containing 0.05% Tween 20 (Sigma). Diluted ETc antiserum (1:1000) was applied for 48 hours at 4°C. Sections were then washed with PBS and incubated with biotinylated antibody to rabbit IgG (Vector) (1:100) for 1 hour at 37°C followed by the incubation with avidin-biotinyl horseradish peroxidase (HRP) complex (Vector) (1:100) for 1 hour at 37°C. The HRP reaction was performed with 0.05% 3,3′-diaminobenzidine and 0.0015% H_2O_2 for 5 min. Bars, 50 μm.

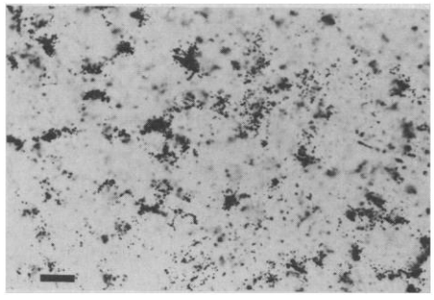

Fig. 3. Autoradiograms of in situ hybridization in the porcine hypothalamic PVN with a ^{35}S-labeled human ET-1 cRNA probe. Porcine hypothalamic PVN regions were obtained as in Fig. 1. Brain tissues were immersed in 4% paraformaldehyde in 0.1*M* borate buffer (*p*H 9.6) for 6 hours and washed in 0.1*M* PBS containing 15% sucrose for 18 hours. Tissues were then frontally sectioned at 20 μm with a cryostat. Sections were mounted on poly-L-lysine–coated slides. A clone that contained a cDNA fragment encoding the 3′ noncoding region of human ET-1 cDNA (phET9I) (*20*) was used as a source of cDNA for the preparation of the cRNA probe. The Eco RI fragment (approximately 700 nucleotides) of phET9I was inserted into the polylinker region of the Bluescript M13 KS$^+$ plasmid (Stratagene) and was cloned (pT7hET9I). The labeled sense or antisense probe was synthesized by incubating the Bam HI linearized recombinant plasmid with either T3 or T7 RNA polymerase (Bethesda Research Labs) in the presence of ^{35}S-labeled cytidine triphosphate (Amersham). Since the 3′ noncoding sequences of human and porcine preproendothelin-1 mRNAs retain nucleotide identity of 77% (*20*), porcine preproendothelin-1 mRNA can readily cross-hybridize with the human ET-1 cRNA probe under the stringency of the in situ hybridization. The transcription procedure and the tissue processing for in situ hybridization was previously described (*21*). Specificity of the labeling was assessed by incubation of the section with a sense-strand probe or treatment of the section with ribonuclease before hybridization (*22*). Bar, 25 μm.

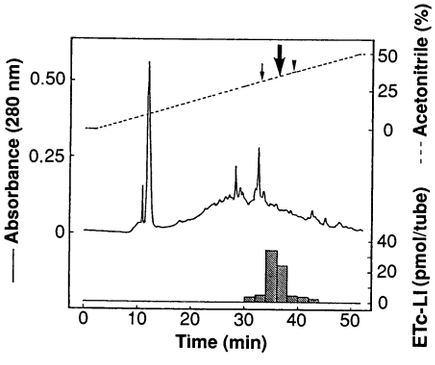

Fig. 2. Chemical identification of ET in the porcine hypothalamus. Porcine hypothalamic regions (total 170 g) were excised from fresh porcine brains and homogenized in two volumes (v/w) of acetone-1*N* HCl (100:3, v/v). The homogenate was stirred at 4°C for 12 hours and centrifuged at 900*g* for 30 min. The supernatant was filtered and washed with a mixture of petroleum ether and diethyl ether (1:1, v/v) and concentrated to 100 ml under vacuum. After ultracentrifugation at 100,000*g* for 60 min, the supernatant was diluted in three volumes with 0.1% trifluoroacetic acid (TFA). This crude extract was applied on a chemcosorb 5ODS-H column (Chemco, 10 by 300 mm). The column was eluted with a 50-min linear gradient from 0 to 50% acetonitrile in 0.1% TFA at a flow rate of 3 ml/min. The effluent was monitored by measuring absorbance at 280 nm. The ET-like immunoreactivity (LI) in each fraction was determined by RIA (*11*). The large arrow indicates the elution time of synthetic ET-1. The small arrow corresponds to the elution position of synthetic ET-3 and the arrowhead corresponds to that of ET-2. The peaks of ET-LI (shaded bars) emerged at the elution position corresponding to that of authentic ET-1.

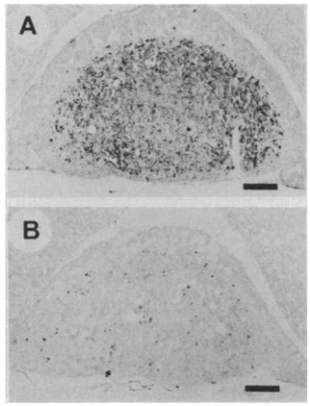

Fig. 4. Depletion of ET-like immunoreactive products in the posterior lobe of the rat pituitary gland after water deprivation. Male Wistar rats weighing about 300 g were used. (**A**) Numerous dot-like immunoreactive products are observed in the posterior pituitary supplied with food and water ad libitum. (**B**) Four days after water deprivation. Body weight decreased from 290 g to 230 g (21% loss). ET-like immunoreactive products in the posterior pituitary are largely depleted. All animals were perfused just before noon. Cryostat sections (10 μm thick) were processed as described in Fig. 1. Bars, 150 μm.

with a sensitive sandwich-type enzyme immunoassay was reported to be around 1.59 pg/ml (*19*), suggesting the presence of the circulating pool of ET. These data are consistent with the possibility that ET is a circulating hormone. Although the contribution of ET in the posterior pituitary system to the circulating level of ET remains unclear, our results suggest that ET has a role in neurosecretion.

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6. [Arg^{14}]ET(15–21) was synthesized with an automatic peptide synthesizer (Applied Biosystems Model 430A) and was coupled to KLH with *m*-maleimidobenzoyl-hydroxysuccinimide ester (*5*). Male New Zealand rabbits were immunized by subcutaneous injection with ETc-coupled KLH in complete Freund's adjuvant (300 μg). Booster injections with the same amount of the antigen were given six times at 2-week intervals. The ETc antiserum was obtained 10 days after the last booster injection. The titer and cross-reactivity of ETc antiserum were evaluated by RIA. ETc antiserum failed to cross-react with the following peptides at more than 10^4-fold molar excess: [Arg^8]vasopressin, [Lys^8]vasopressin, oxytocin, vasopressin-associated neurophysin (human), oxytocin-associated neurophysin (human), [Met^5]enkephalin, [Leu^5]enkephalin, dynorphin A, corticotropin-releasing factor, cholecystokinin octapeptide, neuropeptide Y, angiotensin I (human), and porcine big ET-1.
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10. ET-positive axons from PVN and SON, which passed through the fiber layer of the median eminence and projected to the posterior pituitary, showed varicosities and localized swelling resembling Herring's bodies. In the posterior pituitary, an accumulation of dot-like immunoreactive products for ET was concentrated around the small vessels.
11. For RIA with ETc antiserum, standard ET-1 or unknown samples in 100 μl of RIA buffer [0.05*M* PBS (*p*H 7.4), 0.1% bovine serum albumin, 0.1% Triton X-100, 0.15*M* NaCl, 0.025*M* EDTA · 2 Na, 0.05% NaN_3, and Trasylol (500 kallikrein inactivator units per milliliter)] were previously incubated with diluted ETc antiserum (200 μl, 1:21,250) for 12 hours at 4°C. Then each standard or sample was incubated with ^{125}I-labeled ET-1 (50 μl) (approximately 15,000 cpm) for 24 hours and was then incubated with diluted goat antibodies to rabbit immunoglobulin G (IgG) (500 μl, 1:200) for 30 min at 4°C. After centrifugation, the radioactivity in each precipitate was determined. The median inhibitory concentration (IC_{50}) of tracer binding by ET-1 was observed at 600 fmol per tube. Cross-reactivity of this antiserum with ET-2 or ET-3 is almost the same as with ET-1.
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Mutations of the Adenylyl Cyclase Gene That Block RAS Function in *Saccharomyces cerevisiae*

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The interaction between RAS proteins and adenylyl cyclase was studied by using dominant interfering mutations of adenylyl cyclase from the yeast *Saccharomyces cerevisiae*. RAS proteins activate adenylyl cyclase in this organism. A plasmid expressing a catalytically inactive adenylyl cyclase was found to interfere dominantly with this activation. The interfering region mapped to the leucine-rich repeat region of adenylyl cyclase, which is homologous to domains present in several other proteins and is thought to participate in protein-protein interactions.

DOMINANT INTERFERING MUTATIONS can be useful for investigating interactions between components of signal transduction systems (*1*). Such a mutation can inhibit signal outputs by causing the production of a partially functional protein. The protein is functional in that it binds to an appropriate target, but nonfunctional in that the binding event is not productive. Thus, the mutant molecule sequesters its partner and prevents it from interacting with other components of the system. The net result is a diminished output from the signaling system.

RAS proteins activate adenylyl cyclase in *Saccharomyces cerevisiae* (*2, 3*), and several dominant interfering mutations of *RAS* have been found (*4–7*). One of these mutant proteins (*7*) appears to act by sequestering an "upstream" component, the *CDC25* gene product, which is thought to be an activator of yeast RAS. Feig and Cooper (*5*) isolated a similar mammalian *ras* mutation that appears to interfere with activation of mammalian Ras. Michaeli *et al.* (*6*) demonstrated that mutant RAS proteins that fail to translocate from the cytoplasm to the membrane also

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