Concomitant Expression of Endothelin Receptor Subtype and Isopeptide by Pheochromocytoma and Bovine Adrenal Medulla

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Endothelin (ET) isopeptide and receptor subtype are distributed in various tissues, suggesting diverse functions as local factors other than acting as potent vasoconstrictors. We have studied whether ET isopeptide and receptor subtype are concomitantly expressed in pheochromocytoma and bovine adrenal medulla by binding studies and Northern blot analysis and compared the results with those in human and bovine adrenal cortex. Binding studies using [¹²⁵I]ET-1 as a radioligand revealed a single class of high-affinity binding sites for ET-1 in membranes from two pheochromocytomas and normal bovine adrenal medulla, which were almost comparable to those from human and bovine adrenal cortex. ET-1 and ET-2 equipotently and competitively inhibited [¹²⁵I]ET-1 binding to the membranes, whereas ET-3 was far less potent than ET-1 at higher concentrations, suggesting heterogeneous subpopulations of ET receptors. Northern blot analysis using cDNAs for ET_A and ET_B receptors revealed that both ET_A and ET_B receptor mRNAs are expressed in pheochromocytoma and bovine adrenal medulla in a similar fashion as in human and bovine adrenal cortex. Northern blot analysis using cDNAs for human preproET-1 and preproET-3 as probes also revealed the concomitant expression of preproET-1 mRNA in pheochromocytoma and bovine adrenal medulla and preproET-3 mRNA in human adrenal cortex. These data demonstrate for the first time that ET receptor subtype (ET_A and ET_B) and ET isopeptide (ET-1 and ET-3) are concomitantly expressed by pheochromocytoma as well as bovine adrenal medulla, suggesting the paracrine/autocrine function of ET in adrenomedullary chromaffin cells. (*Hypertens Res* 1993;16: 215-219)

Key Words: endothelin, receptor subtype, isopeptide, pheochromocytoma, adrenal medulla

Endothelin (ET) is a potent vasoconstrictor peptide with a 21-amino-acid residue, originally isolated from porcine endothelial cells (1). Subsequent cDNA cloning from human genomic library revealed the existence of three distinct isopeptides, termed ET-1, ET-2 and ET-3 (2). These three isopeptide mRNAs have been shown to be distributed in a variety of tissues of human and other species. Recently, at least two similar, but distinct ET receptors, designated ET_A and ET_B subtype, have been cloned and sequenced (3,4); ET_A receptor is selective for ET-1 and ET-2, while ET_B receptor is non-isopeptide-selective. These two receptor subtypes have also been shown to be distributed in various tissues where the three isopeptides are colocalized. These observations led to the suggestion that ET isopeptides may function as an autocrine/paracrine factor in various tissues.

It has been reported that ET-1 stimulates hormone secretion from various endocrine glands, including atrial natriuretic peptide from rat cardiocytes (5), gonadotropins from rat pituitary gland

(6), vasopressin from rat hypothalamus (7), aldosterone from adrenal zona glomerulosa cells (8), and catecholamines from bovine adrenomedullary chromaffin cells (9). However, there is currently no evidence available demonstrating whether or not ET isopeptide and its receptor subtype are concomitantly present in human and bovine adrenal medulla. Therefore, the present investigation using binding study and Northern blot analysis was designed to elucidate whether ET isopeptide and its receptor subtype are concomitantly expressed by pheochromocytoma and bovine adrenal medulla and to compare the results with those in human and bovine adrenal cortex.

Methods

Patients

Case 1: A 32-year-old male was found to have a right adrenal incidentaloma (2.2 × 2.3 × 2.0 cm) by CT scan and magnetic resonance imaging. He had been normotensive (120/60 mmHg) without any epi-

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sodes of paroxysmal hypertension. 24-h urinary excretions of vanil mandelic acid, norepinephrine, epinephrine and dopamine were 6.2 mg, 206 μ g, 14 μ g and 919 μ g, respectively.

Case 2: A 47-year-old male with a paroxysmal hypertensive attack (200/100 mmHg) was found to have a left adrenal mass ($3.7 \times 2.5 \times 3.5$ cm) by CT scan and magnetic resonance imaging. 24-h urinary excretions of vanil mandelic acid, norepinephrine, epinephrine and dopamine were 16.4 mg, 2036 μ g, 119 μ g and 1943 μ g, respectively.

Both cases underwent surgical removal of the adrenal tumors. Pathologically the tumor specimens from both cases were compatible with the diagnosis of pheochromocytoma. Their operative and postoperative courses were uneventful.

Tissue Samples

Tumor tissues from the two cases with pheochromocytoma were obtained during surgery. Adjacent normal cortical tissue was separated from the tumor tissue of Case 2. Bovine adrenal glands obtained at a local slaughterhouse were dissected into cortical and medullary portions. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Binding Study

Adrenal tissue specimens were homogenized by a Polytron homogenizer in 20 mM phosphate buffer, pH 7.5, (130 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin), and centrifuged at $15,000 \times g$ for 5 min. The pellets were resuspended in the same buffer and used as membrane preparations. For binding studies, membrane suspensions (10–50 μ g protein) were incubated with 25 pM [^{125}I]ET-1 (Amersham Japan, Tokyo) in the absence and the presence of various concentrations of unlabeled ET-1, ET-2 and ET-3 (Peptide Institute Inc., Osaka) at 30°C for 30 min in the same manner as recently reported (10). Incubation was terminated by the removal of free radioactive ligands after centrifugation. Specific binding was determined by subtracting nonspecific binding in the presence of excess (10^{-7} M) unlabeled ET-1 from total binding.

Northern Blot Analysis

RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (11). Briefly, frozen tissue specimens from Case 2 and bovine adrenal glands were homogenized in guanidinium solution (10 ml/g tissue: 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 1% 2-mercaptoethanol) using a Polytron homogenizer. One ml 2 M sodium acetate (pH 4.0), 10 ml water-saturated phenol, and 2 ml chloroform-isoamyl alcohol mixture (49 : 1) were added to the homogenates with thorough mixing and vortexing, and the mixture was cooled on ice for 15 min. After centrifugation at $10,000 \times g$ for 20 min at 4°C , an aqueous phase containing RNA was precipitated twice with 10 ml isopropyl alcohol at -20°C for

20 min. Total RNA was pelleted by centrifugation, washed with 70% ethanol, dissolved in diethylpyrocarbonate-treated water, and subjected to poly(A)⁺ selection by oligo(dT) chromatography. Poly(A)⁺ RNA (10 μ g) was separated by formaldehyde/1.1% agarose gel electrophoresis and transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westborough, MA). RNA immobilized on the membrane was hybridized respectively with cloned cDNAs for human and bovine ET_A and ET_B receptors and those for preproET-1 and preproET-3 as probes, in the presence of 50% formamide at 42°C over 16 h. The cDNA inserts of human ET_A and ET_B receptor (12), bovine ET_A receptor (3) and ET_B receptor (13), human preproET-1 and preproET-3 (10), and bovine preproET-1 (14) were labeled with deoxycytidine 5'-[^{32}P]triphosphate (111 TBq/mmol, DuPont NEN Research Products, Boston, MA) by the random-primed labeling method. The membranes were washed finally in $0.1 \times \text{SSPE}$ (15 mM NaCl, 1 mM NaH_2PO_4 , 0.1 mM ethylenediaminetetraacetic acid) / 0.5% sodium dodecyl sulfate at 50°C , and autoradiographed with intensifying screens at -80°C for 20–24 h.

Results

Equilibrium binding of [^{125}I]ET-1 to the membrane preparations from human and bovine adrenal glands was reached after 30 min at 30°C ; non-specific binding was less than 10% of total binding.

Competitive bindings of [^{125}I]ET-1 to the membranes from two pheochromocytomas (Cases 1 and 2) by unlabeled isopeptides are shown in Fig. 1. ET-1 competitively inhibited the binding of [^{125}I]ET-1 to the membranes in both cases. Scatchard analysis (Inset) revealed the presence of a single class of high-affinity binding sites for ET-1; the apparent dissociation constants (K_d) were 3.7×10^{-11} M (Case 1) and 3.5×10^{-10} M (Case 2), and the maximal binding capacities (B_{max}) were 130 fmol/mg protein (Case 1) and 145 fmol/mg protein (Case 2), respectively. ET-2 was about 5-fold less potent than ET-1 to displace [^{125}I]ET-1 from its binding sites, whereas ET-3 was far less potent than ET-1 and ET-2 with the apparent inhibition constants (K_i) of 2.2×10^{-8} M (Case 1) and 4.5×10^{-8} M (Case 2), respectively.

Competitive binding studies using membranes of normal bovine adrenal cortex and medulla are shown in Fig. 2. ET-1 competitively inhibited the binding of [^{125}I]ET-1 to the membranes from bovine adrenal cortex and medulla; the apparent K_d s in bovine adrenal cortex and medulla were 3.5×10^{-11} M and 4.5×10^{-11} M, respectively, and B_{max} s were 69 fmol/mg and 55 fmol/mg, respectively. ET-2 was almost as potent as ET-1 in adrenal cortex and about four-fold less potent than ET-1 in adrenal medulla, whereas ET-3 was far less potent than ET-1 to displace [^{125}I]ET-1 from its binding sites in both adrenal cortex and medulla at higher concentrations (10^{-9} – 10^{-7} M). The same binding characteristics were noted in the membrane from human adrenal cortex from Case 2 (data not shown).

Northern blot analysis using cDNAs for human

Fig. 1. Competitive binding of [125 I]ET-1 by ET isopeptide to pheochromocytomas. Membranes from two pheochromocytomas (Cases 1 and 2) were incubated with [125 I]ET-1 at 30°C for 30 min in the absence and presence of unlabeled ET-1 (●), Et-2 (▲) and Et-3 (■) in the indicated concentrations. Each point is the mean of triplicate samples. (Inset) Scatchard plots of ET-1 binding.

Fig. 2. Competitive binding of [125 I]ET-1 by ET isopeptide to bovine adrenal cortex and medulla. Membranes from bovine adrenal cortex (1) and medulla (2) were incubated with [125 I]ET-1 at 37°C for 30 min in the absence and presence of unlabeled ET-1 (●), ET-2 (▲) and ET-3 (■) in the indicated concentrations. Data are plotted as in Fig. 1.

ET_A and ET_B receptors and human preproET-1 and preproET-3 as probes in pheochromocytoma and adrenal cortex from Case 2 is shown in Fig. 3. Two distinct bands (4.3 kb and 4.8 kb) corresponding to the sizes of human ET_A and ET_B mRNA were observed in both pheochromocytoma and adrenal cortex. Two distinct bands (2.3 kb and 2.8 kb) corresponding to the sizes of human preproET-1 and preproET-3 mRNA were noted in pheochromocytoma

and adrenal cortex; preproET-1 mRNA was evident in pheochromocytoma, whereas preproET-3 mRNA was predominant in adrenal cortex.

Northern blot analysis using cDNAs for bovine preproET-1 as probes in bovine adrenal cortex and medulla is shown in Fig. 4. Two bands (4.3 kb and 3.3 kb) corresponding to the sizes of bovine ET_A mRNA and a single band (4.8 kb) corresponding to the size of bovine ET_B mRNA were observed in

Fig. 3. Northern blot analysis of human ET receptor subtype and isopeptide in pheochromocytoma and adrenal cortex. Poly (A)⁺ RNA (10 μ g) from pheochromocytoma (P) and adjacent adrenal cortex (C) from Case 2 was subjected to Northern blot using [³²P]cDNAs for human ET_A and ET_B, preproET-1 and preproET-3 as probes, respectively. Arrows show 28S and 18S ribosomal RNA.

Fig. 4. Northern blot analysis of bovine ET receptor subtype and preproET-1 in bovine adrenal cortex and medulla. Poly (A)⁺ RNA (10 μ g) from normal bovine adrenal cortex (C) and medulla (M) was subjected to Northern blot using [³²P]cDNAs for bovine ET_A, ET_B and preproET-1 as probes, respectively.

both adrenal cortex and medulla. A single band (2.3 kb) corresponding to the size of bovine preproET-1 mRNA was distinctly noted in cortical tissue, but only faintly in medullary tissue.

Discussion

It has been shown that ET-1 stimulates release of aldosterone from rat adrenal zona glomerulosa cells (8) and catecholamines from bovine adrenomedullary chromaffin cells (9), suggesting that it acts as a local regulator of hormone secretion in adrenal glands. Recently, Wilkes and Broader (15) have reported that cultured bovine adrenomedullary chromaffin cells possess non-isopeptide-selective ET receptor, possibly ET_B subtype, as assessed by binding studies. The present competitive binding studies clearly demonstrated that two pheochromocytomas, human adrenal tumors derived from adrenomedullary chromaffin cells, possessed a single class of binding sites for ET-1 with high affinities (K_d : 10^{-11} – 10^{-10} M) and capacities (B_{max} : 130–145 fmol/mg protein), almost comparable to those of normal bovine adrenal medulla. In the present binding studies, however, ET-3 produced biphasic competition binding curves against [¹²⁵I]ET-1 in the

membranes from pheochromocytomas and bovine adrenal medulla. These data are consistent with the existence of heterogeneous populations of ET receptors in these adrenal tissues. Northern blot analysis using cDNAs for human ET_A and ET_B receptors as probes revealed concomitant expression of ET_A and ET_B receptor mRNAs in pheochromocytoma and bovine adrenal medulla, thus verifying the existence of two distinct ET receptor subtypes in adrenomedullary tissues. The co-expression of ET_A and ET_B receptor mRNAs by human as well as bovine adrenocortical tissues as demonstrated in this study is consistent with our previous observations (10). The reason why Wilkens and Boarder (15) could not demonstrate the presence of ET_A receptors in bovine adrenal chromaffin cells remains unknown. Possibly, digestion of adrenomedullary chromaffin cells by collagenase/protease and/or the culture conditions employed in their study may have led to preferential loss of ET_A receptor.

The present Northern blot analysis using cDNAs for human preproET-1 and preproET-3 as probes further demonstrated the expression of both preproET-1 and preproET-3 mRNA in human adrenal tissues; preproET-1 mRNA and preproET-3 mRNA were predominant in pheochromocytoma

and in the adjacent adrenocortical tissue, respectively. This is in contrast to the predominant expression of preproET-1 mRNA in bovine adrenal cortex as demonstrated by Northern blot analysis. Species differences and/or malignant transformation of adrenal chromaffin cells may partly account for the different expression of ET isopeptide. Our data confirm our previous observation that ET isopeptide and receptor subtype are concomitantly expressed by human adrenocortical tissues and adenomas (10), and further extend the concomitant expression of ET isopeptide and receptor subtype by adrenal medulla and pheochromocytoma.

The physiological function of ET isopeptide in adrenal medulla remains obscure. ET-1 has mitogenic action on a number of cells, including vascular smooth muscle cells (16), glomerular mesangial cells (17), and certain human tumor cells (18). Therefore, it is possible to speculate that ET-1, in addition to its stimulatory effect on catecholamine secretion (15), may affect growth and/or differentiation of adrenal chromaffin cells as an autocrine/paracrine factor. The pathophysiological significance of the ET family in relation to tumor growth of pheochromocytoma thus needs to be determined.

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