Endothelin: Visualization of mRNAs by in situ hybridization provides evidence for local action
(regulatory peptide/vasoconstrictor/vascular endothelium/cardiovascular control/neuromodulator)

MATTHEW W. MACCUMBER*†, CHRISTOPHER A. ROSS*‡, BERT M. GLASER¶, AND SOLOMON H. SNYDER*¶§

Departments of *Neuroscience, Pharmacology and Molecular Sciences, †Psychiatry and Behavioral Sciences, and ¶Ophthalmology, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205; and §The Retina Center at Saint Joseph Hospital, Towson, MD 21284

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ABSTRACT

Endothelin (ET) is a recently identified vasoactive peptide with three isoforms for which three genes have been cloned. The cellular sites of synthesis of this peptide have not yet been identified in vivo. Using Northern blot analysis, we have detected two forms of ET mRNA in rat tissues: a 3.7-kilobase form in the kidney, eye, and brain, a 2.5-kilobase form in the intestine, and both forms in the lung. We have localized these forms of ET mRNA in several rat tissues using in situ hybridization. In the 19-day rat fetus, ET mRNA is highest in the lung, intestine, and meninges. At high resolution, ET mRNA is localized in the lung to respiratory epithelial cells of bronchioles and apparently in blood vessels. In adult tissues, ET mRNA is present throughout the lung, in the renal medulla, and in the iris of the eye. ET mRNA is synthesized in close proximity to ET binding sites in many organs (e.g., lung, kidney, intestine, and eye), suggesting a local action of this peptide. However, in other areas (e.g., heart and renal cortex), ET binding sites are present in the absence of ET mRNA, suggesting an action of ET from the bloodstream or from neurons. Northern blot analysis of ET mRNA in microvascular endothelial cells in culture indicates that ET is synthesized in small blood vessels and regulated similarly to its regulation in large vessels. Our results provide evidence that ET, like other regulatory peptides, may serve in several tissues as a neuromodulator or local hormone.

Endothelin (ET) is a 21-amino acid peptide isolated from aortic endothelial cells in culture and shown to be an extremely potent vasoconstrictor (1). Genes for three forms of ET have been identified in the human and rat and have been cloned from a human genomic library (2). However, mRNA for only one of these forms has been identified and only in endothelial cells from aorta or umbilical vein (1, 11). In aortic endothelial cells, levels of mRNA for ET-1 are regulated by several hormonal substances or analogues (1). In peripheral tissues, ET receptor binding sites have been identified primarily in smooth muscle, especially of blood vessels and airways (3–5). Because ET is relatively resistant to peptidases, it has been suggested that ET functions primarily as a circulating hormone, while an alternative model is for ET to be elaborated by endothelial cells adjacent to the muscle cells on which it acts. However, the cellular sites for biosynthesis of ET have not yet been identified in any tissue in vivo. In the present study we have localized sites of ET formation by in situ hybridization techniques. We provide evidence that in most parts of the body ET is formed near the site of its action and also demonstrate dynamic regulation of ET mRNA in microvessel endothelial cells.

METHODS

Two oligonucleotide probes (39 bases long) were synthesized based on the published rat ET-3 sequence (6) and purified by HPLC. Sequences were chosen to avoid internal repeats, regions with known homology to other sequences, and regions with greater than expected homology to rat structural RNA. For each sequence, complementary (antisense) and identical (sense) probes were synthesized. The probe sequences are designated "A" (for the sense probe, bases 1–39, 5'-CACCGACCTCGGCATGCTTCATCTATAT-3') and "B" (for the sense probe, bases 59–97, 5'-CTACTGCCAAGATCATGCTGGATCAC-3'). If it is assumed that the three rat ET sequences are highly homologous to those of human ET (2), probe A would be expected to strongly hybridize to ET-3 and only weakly to ET-1 and ET-2, whereas probe B would hybridize strongly to ET-1 and ET-3 and only weakly to ET-2 in our hybridization and wash conditions.

In situ hybridization was performed using minor modifications (7) of the procedure of Young et al. (8). Briefly, probes were 3'-end-labeled using [α-32P]dATP or deoxyadenosine 5'-[α-32P]dATP or deoxynucleotide transferase to a specific activity of ≈10⁵ Ci/mmol (for 35S) or 5 × 10⁴ Ci/mmol (for 32P) (1 Ci = 37 Gbq), and incubated overnight in hybridization buffer containing 50% (vol/vol) formamide and 4× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.4). High-stringency washes the next day were at 55°C in 1× SSC/5% formamide for 1 hr. Labeled sections were apposed to β-particle-sensitive film (Amersham) for 3–7 weeks.

Some sections (labeled with 35S) were dipped in Kodak NTB2 emulsion diluted 1:1 in water, exposed for 5–10 weeks, and developed in D19.

For Northern blots, probes were labeled as above with [α-32P]dATP. Total RNA (20 µg per well) was fractionated in a 1% agarose gel and transferred to nylon membranes. Stringency conditions for hybridization and washes of the blots were exactly the same as for in situ experiments.

125I-labeled ET-1 (see Fig. 1B) was prepared by iodination of the ET-1 peptide (10 µg; Peptides International, Louisville, KY) in 50 mM Tris-HCl (pH 7.4, 100 µl) containing two washed Iodo-Beads (Pierce) and 1 mCi of carrier-free Na125I (17 Ci/mol; NEN/DuPont) for 30 min with purification by gel filtration (∼5 Ci/mmol). For all other binding experiments, 125I-labeled ET-1 was obtained from NEN/DuPont (2200 Ci/mmol). Repeat of Fig. 1B with the higher specific activity ligand gave a similar picture to that shown.

For binding experiments, 125I-labeled ET-1 (30,000 cpm) was added to 12-µm fresh-frozen cryostat-cut sections or

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Abbreviation: ET, endothelin.
"To whom reprint requests should be addressed.

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tissue homogenates in 50 mM Tris-HCl, pH 7.4/150 mM NaCl/0.1% bovine serum albumin for 1 hr. Unlabeled ET-1 (1 μM) was added for blanks. Homogenates were washed twice with 50 mM NaCl and radioactivity was measured after rapid filtration (Brandel, Gaithersburg, MD). Sections were washed for three 20-min periods in the incubation buffer, dipped quickly in distilled H2O, fixed with 4% (wt/vol) paraformaldehyde/1% glutaraldehyde, and apposed to film or dipped in Kodak NTB2 emulsion and exposed for 1-3 days.

Human retinal microvessel endothelial (HME) cells were isolated by published procedures (9) and grown to confluence in modified Eagle’s medium (MEM) with 15% (vol/vol) fetal calf serum (GIBCO). The cells were washed twice in balanced salt solution and then incubated in MEM plus 0.1% bovine serum albumin with or without drugs for 2 hr. Transforming growth factor β (type 2) was obtained from R & D Systems (Minneapolis). At 2 hr, cells were treated with trypsin and RNA was prepared for Northern blot analysis. Biots were probed using an oligonucleotide probe (45 bases long) based on the published sequence bases 175-219 for human ET-1 (10) and end-labeled as above using [α-32P]dATP and terminal deoxynucleotidyltransferase. Autoradiograms were quantified using an image analysis system (Amersham).

RESULTS

Northern Blot Analysis of ET mRNA. We conducted Northern analysis of several rat tissues utilizing oligonucleotide probe B based on the published sequence for the rat form of ET-3 (6) (Fig. 1). If we assume the rat ET sequences differ from one another to the extent that the human ET sequences differ (2), probe A would detect ET-3 only, and probe B would detect both ET-1 and ET-3. We observed two transcripts of ~3.7 and 2.5 kilobases (kb). In the lung we observed both bands, although the 2.5-kb band was more intense than the 3.7-kb band. The eye, kidney, cerebellum (Fig. 1), and other brain regions (data not shown) displayed only single bands at 3.7 kb, but the eye and kidney were

Fig. 2. ET mRNA localized by in situ hybridization and ET binding sites in the 19-day rat fetus. (A) In situ hybridization. (B) ET binding sites. (C) In situ hybridization using the “sense” orientation probes. (D) Adjacent section to that in A stained with cresyl violet. Cb, cerebellum; H, heart; I, intestine; Li, liver; Lu, lung; M, meninges; NM, nasal mucosa; P, penis. (Bar = 5 mm.)
substantially more intense than any brain region. The intestine displayed only one band at 2.5 kb. No bands were visualized in liver or right atrium of the heart.

Utilizing a probe selective for ET-3 (probe A), we observed only a single band at 3.7 kb in the same tissues as shown in Fig. 1 (data not shown). In porcine (1) and human (11) vascular endothelial cells ET-1 mRNA is ≈2.5 kb. Thus, we assume that the 2.5-kb transcript represents ET-1 and the 3.7-kb transcript reflects ET-3, so that kidney, eye, and brain predominantly express ET-3 while lung expresses both ET-1 and ET-3.

**Comparative Localizations of ET mRNA and Binding Sites in Fetal Rats.** To assess the localization of ET mRNA and ET binding sites throughout the body, we conducted in situ hybridization for ET mRNA and autoradiography for ET binding sites in the whole 19-day rat fetus (Fig. 2).

In situ hybridization employed a mixture of equal amounts of the two oligonucleotide probes labeled with [35S] by terminal deoxynucleotidyltransferase. The specificity of the technique was shown by the detection of little to no signal in the lung and only a light signal in the liver and fat with "sense" orientation probes for both sequences (Fig. 2C). Also, the antisense labeling was abolished by pretreatment of sections with RNase, the addition of 100-fold excess unlabeled antisense probe, or washes 15–10°C higher than the melting temperature (data not shown).

Receptor binding autoradiography utilized [125I]-labeled ET-1. In homogenates of various tissues, we demonstrated saturable binding of [125I]-labeled ET-1 with a $K_d$ of ≈0.5 nM with abundant binding in heart and lung and somewhat less in kidney, similar to other reports (4, 12) (data not shown). Binding was abolished in the fetus sections in all locations except bone by 1 µM unlabeled ET-1 (data not shown).

ET mRNA and binding sites displayed similar localizations with highest levels in the lung (Fig. 2). Moderate levels of both mRNA and binding sites were detected in intestine, meninges, and nasal mucosa. Lowest levels of mRNA and binding sites were in the liver. The heart and the developing cerebellum contained high levels of ET binding but low levels of mRNA.

In the lung at high resolution, in situ silver grains were intimately associated with small bronchioles especially over the respiratory epithelial cells (see Fig. 4). Grains were also present in a patchy distribution over lung parenchyma but not associated with bronchioles, apparently over small vessels.

**Localization of ET mRNA and Binding Sites in Adult Rat Organs.** We localized ET mRNA by in situ hybridization and ET binding sites by autoradiography in adult rat organs (Figs. 3 and 4). In the lung, at low resolution, mRNA and receptors were widely distributed. Renal ET binding sites and mRNA were both localized to the medulla with a distribution indicating an association with the vasa recta. In the cortex, ET binding sites were observed in the glomeruli, which failed to display mRNA. In the eye, ET binding sites and mRNA were highly concentrated in the iris, where they were localized to the stroma, which is greatly enriched in small blood vessels. High densities of ET binding sites were also observed in the cornea in the endothelial layer and Descemet's membrane, the choroid, and the retina (Fig. 4). No mRNA was apparent in the cornea (grains seen near the cornea in Fig. 4D are an

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**Fig. 3.** ET mRNA localized by in situ hybridization (A, C, and E) and ET binding sites (B, D, and F) in adult rat tissues. (A and B) Kidney. (C and D) Lung. (E and F) Eye. Labeling of lens (L) and orbital fat (OF) is nonspecific. B, bronchus; C, renal cortex; Ch, choroid; CE/D, corneal endothelium/Descemet's membrane; G, glomeruli; I, iris; L, lens; M, renal medulla; OF, orbital fat; P, renal pelvis; R, retina; and VR, vasa recta. (Bars = 5 mm.)
edge artifact), and only low levels were apparent in the choroid. Only low levels of ET mRNA or binding sites were present in any part of the ciliary body. Label for either ET mRNA or binding sites in orbital fat and the lens was nonspecific.

**Regulation of ET mRNA Levels.** Yanagisawa et al. (1, 11) showed changes in ET mRNA levels in aortic and umbilical vein endothelial cells in culture in response to various stimuli. For example, phorbol esters transiently increased and then decreased ET-1 mRNA (11). Large vessels such as the aorta and umbilical vein differ from the microvasculature in various regulatory mechanisms. Our *in situ* hybridization studies indicated an association of ET with small blood vessels in the iris, kidney, and probably the lung. Accordingly, we explored the influence of various substances on ET mRNA analyzed by Northern blot in endothelial cell cultures from microvessels of the human retina (Fig. 5). Consistent with the observations of Yanagisawa et al. (11), we observed a decline of ET mRNA 2 hr after treatment with 1 μM phorbol 12,13-dibutyrate, a phorbol ester. To assess whether this effect was due to an interaction with protein kinase C, we pretreated some preparations with the protein kinase C inhibitor H7 (250 μM), which partially blocked the effect of phorbol 12,13-dibutyrate. H7 alone caused a small decrease in ET mRNA levels. We found similar effects of phorbol esters and H7 on fetal bovine aortic endothelial cells (data not shown). We also observed increases in ET mRNA concentration in preparations treated with 1 μM phenylephrine (75% increase, Fig. 5) and transforming growth factor β (type 2) at 5 ng/ml (60% increase, data not shown).

**DISCUSSION**

The present study has identified ET mRNA both by Northern blot analysis and *in situ* hybridization in a variety of tissues of the fetal and adult rat. ET mRNA had been detected only in the aorta and umbilical vein (1, 11). We have demonstrated ET mRNA by Northern blot analysis in several tissues. Moreover, we observed expression of different forms of ET in various tissues. Thus, the intestine displays the 2.5-kb transcript (presumably ET-1), the kidney, eye, and brain possess the 3.7-kb transcript (presumably ET-3), while the lung displays both ET-1 and ET-3. Lacking a nucleotide sequence for rat ET-2, we were unable to construct an oligonucleotide probe to explore for its presence. The functional role of the different types of ET is unclear. Conceivably, tissues with more than one subtype of ET might express the different types in different tissue constituents. For example, one form may be produced by respiratory epithelial cells and another by pulmonary microvessels.

A major finding of this study is the similarity in localization of ET mRNA and binding sites in various organs. It seems likely that in these organs ET functions as a local hormone,
being elaborated in epithelial or endothelial cells and acting on adjacent smooth muscle.

In the lung, for example, mRNA and binding sites are widespread. However, at high resolution, synthesis of ET mRNA appears localized to the respiratory epithelium of bronchioles and has a patchy distribution elsewhere. Either aerosolized or intravenous ET causes marked bronchoconstriction (13, 14), suggesting that ET synthesized in respiratory epithelium acts on the nearby airway smooth muscle. The patchy distribution of ET apart from the bronchioles suggests that ET may be synthesized not by capillaries but by larger microvessels surrounded by smooth muscle.

In the eye, ET mRNA and binding sites are both localized to the iris; however, prominent ET binding sites also occur in the corneal endothelium. Perhaps the ET elaborated in the iris passes through the aqueous humor to influence receptors on the corneal endothelium in addition to adjacent receptors in the iris.

In the kidney, mRNA and binding sites are localized to the renal medulla. ET binding sites are also prominent in glomeruli of the renal cortex, where we cannot detect ET mRNA. ET acting on glomerular receptors may come from the bloodstream or autonomic innervation. We have observed an mRNA that hybridizes to probe B neuronal cells of the superior cervical ganglion (unpublished data). Alternatively, a form of ET (i.e., ET-2) not detected by our probes may be synthesized locally in the glomeruli.

ET binding sites are also prominent in cardiac ventricular muscle in the absence of ET mRNA. Autonomic innervation of this tissue or the bloodstream may be the source of ET or ET-2 mRNA may be synthesized in the heart.

A neural localization for ET is suggested by the ET mRNA in the cerebellum (Fig. 1) and other brain regions (unpublished data) where it may have a neurotransmitter or neuromodulator action to stimulate inositol phospholipid turnover (12). Like other neuropeptides ET may serve in some tissues as a local hormone and in others as a neurotransmitter or neuromodulator.

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