Localization of the Endothelin System in Aldosterone-Producing Adenomas

Giorgia Egidy, Erno Baviera, Gladys Ciuffo, Pierre Corvol, Florence Pinet

Abstract—Endothelin-1 (ET-1) could play a role in the regulation of aldosterone secretion of the human adrenal gland. The presence of the endothelin-converting enzyme 1 (ECE-1) and ET-1 suggests that there is a local ET system in the adrenal cortex, but the in situ synthesis of ET-1 remains to be confirmed. The cellular distribution of the whole ET system was evaluated in 20 cases of aldosterone-producing adenomas. Polymerase chain reaction studies gave strong signals for ECE-1 mRNA and the mRNAs for endothelin type A (ET_A) and B (ET_B) receptors and faint signals for prepro–ET-1 mRNA. In situ hybridization showed ET_A receptors scattered throughout the adenoma, in both secretory cells and vascular structures (score, +). There were more ET_B receptors (score, ++), but they were restricted mainly to the endothelium. ECE-1 mRNA and protein were ubiquitous and abundant in secretory cells (score, +++) and vascular structures (score, ++); the enzyme was active on big ET-1. There was no prepro–ET-1 mRNA in the cortex, except in the thickened precapillary arterioles present in only 30% of the aldosterone-producing adenomas studied. ET-1 immunoreactivity was detected in vascular structures (score, +), probably bound to receptors, suggesting that ET-1 has an endocrine action. The low concentrations of ET-1 could also indicate that it acts in a paracrine-autocrine fashion to control adrenal blood flow. The discrepancy between the concentrations of ECE-1 and its substrate suggests that ECE-1 has another role in the adrenal secretory cells. Our data indicate that ET probably is not a primary cause of the development or maintenance of the adenoma. **(*Hypertension*. 2001;38:1137-1142.)**

Key Words: endothelin ■ hypertension, endocrine ■ receptors, endothelin ■ regional blood flow ■ immunohistochemistry

Endothelin-1 (ET-1) is one of three 21–amino acid peptides, the ETs (ET-1, ET-2, and ET-3), that mediate a broad spectrum of biologic actions, such as the regulation of endocrine secretion.[1] It also is involved in specific vascular lesions and inflammatory conditions (primary and malignant hypertension).[2] The ETs act on 2 distinct high-affinity G protein–coupled receptors, endothelin type A (ET_A)[3] and type B (ET_B),[4] and are synthesized as large precursor polypeptides, the prepro-ETs, which are cleaved at 2 pairs of basic amino acids to generate intermediate peptides, the big ETs. The big ETs are then cleaved by an endothelin-converting enzyme (ECE), initially purified from bovine adrenal cortex,[5] to produce the mature ETs. Two human ECE genes have been cloned, *ECE-1*[6] and *ECE-2*,[7] and they are 59% identical.

ET-1 was first found in the vascular endothelium,[8] but it is also present in endocrine tissues, such as the adrenal gland,[9] where it may mediate the adrenocortical response to ACTH.[10] Large amounts of ECE-1 mRNA and protein have been found in the human adrenal gland, with the highest concentrations in the zona glomerulosa and fasciculata.[11] Both ET receptor subtypes and ET peptides are found in the human adrenal gland.[9] The mRNAs for ET_A, ET_B, and prepro–ET-1 (PPET-1) have also been detected by Northern blotting,[9] RT-PCR,[12] and immunohistochemistry.[13] Quantitative analysis of the binding of ^{125}I-ET-1 by autoradiography together with the use of selective ligands showed that ET_B receptors are present through the entire adrenal cortex, whereas ET_A receptors are restricted to the zona glomerulosa.[14]

Compelling evidence indicates that the ET components in the normal adrenal gland could play a role in the regulation of this gland. Several reports suggest that ET-1 is implicated in steroid hormone secretion. Exogenous ET-1 influenced adrenal function in human volunteers by selectively increasing the ACTH-induced secretion of aldosterone,[15] and a limited study showed that patients with hypertension due to primary aldosteronism had abnormally high levels of plasma ET-1.[16]

Conn's syndrome is a major cause of primary hyperaldosteronism due to an aldosterone-producing adenoma (APA) in 1 adrenal gland. The APA is histogenetically derived from the zona glomerulosa cells,[14] which form the proliferative layer involved in the maintenance of growth of the entire adrenal cortex in mammals and the presence of both ET receptors in APAs.[13,17] Thus, ET-1 may help to regulate the growth of the adrenal cortex.

Received December 15, 2000; first decision December 28, 2000; revision accepted April 23, 2001.

From the INSERM Unit 36, Collège de France (G.E., P.C., F.P.), Paris, France; Service d'Anatamopathologie, Hopital Saint-Joseph (E.B.), Paris, France; and Catedra de Bioquimica Avanzada, Universidad Nacional de San Luis (G.C.), San Luis, Argentina.

Correspondence to Dr Florence Pinet, INSERM Unit 36, Collège de France, 11 place Marcellin Berthelol, 75005 Paris, France. E-mail florence.pinet@college-de-france.fr

Hypertension **is available at http://www.hypertensionaha.org**

This study was conducted to elucidate the pathophysiological significance of ET-1 in APA adrenal glands, as ET-1 is shown to be the predominant mature isoform in the human adrenal gland.[12] We looked for mRNAs that encode all the components of the ET system in human APA and immunoreactive ET-1 and ECE-1 to determine whether there is a complete local ET system in the human adrenal cortex or whether ET-1 is supplied by the blood.

Methods

Human Tissues

Human adrenal tissue was obtained from patients (n=20) undergoing unilateral adrenalectomy for Conn's syndrome by the COMETE network in accordance with French laws. The patients included 11 middle-aged women and 9 middle-aged men. The adenomas weighed 3.1 to 16.8 g. A small piece of each specimen was frozen for RNA extraction, and the remainder was fixed in 4% buffered paraformaldehyde, for ≥24 hours, before processing.

RNA Isolation and RT-PCR

Total RNA was isolated from frozen adrenal cortex according to the protocol of Chomczynski and Sacchi.[18] cDNA was prepared with 5×10^{-6} g total RNA and 15 pmol/L oligo(dT) with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) in 50 μL according to the manufacturer's instructions. PCR was performed using 3 μL cDNA solution and specific primers for ECE-1,[19] PPET-1,[20] ET_A,[3] and ET_B[4] receptors and GAPDH,[21] as previously described.[22] Amplified products were analyzed on 2% agarose gel.

In Situ Hybridization

Radiolabeled riboprobes for PPET-1, ECE-1, ET_A, and ET_B mRNAs were prepared and used with deparaffinized sections (5 μm).[22] The processed sections were exposed for 2 weeks with ECE-1, for 4 weeks with ET_A and ET_B probes, and for 6 weeks with PPET-1 probes and counterstained with toluidine blue.

Immunohistochemistry

Paraffin was removed from embedded sections (5 μm) with xylene, and the sections were rehydrated in a graded ethanol series. Their endogenous peroxidase was inactivated with 3% hydrogen peroxide in methanol for 10 minutes before incubation with monoclonal antibodies to CD31 (DAKO), α-smooth muscle actin (α-SMA) (DAKO), and ET-1 (IHC 6901; Peninsula Laboratories) according to the manufacturer's instructions. The specific antiserum 473-17-A[23] was used for ECE-1. Sections were then reacted with an avidin-biotin complex (ABC; DAKO), treated with 0.035% diaminobenzidine (Fluka) and counterstained with hematoxylin (according to Mayer). Control reactions without primary antibodies showed no nonspecific staining (not shown).

The ET-1 antibody used was specific for human ET-1 but cross-reacted (76%) with human big ET-1 according to the manufacturer. However, we tested the antibody to determine whether it recognized receptor-bound ET-1 by using stable transfected Chinese hamster ovary (CHO) cell lines embedded in paraffin. These were CHO/PPET-1/ECE-1 cells, which secrete human ET-1, and CHO/ET_B cells, which produce human ET_B receptors. Briefly, cells were grown to confluence, scraped off, collected by centrifugation, fixed with 4% paraformaldehyde, and embedded in paraffin. Cultures of CHO/ET_B grown to confluence were incubated with 20 nmol/L ET-1 for 30 minutes at 4°C and processed as described earlier.

The results of in situ hybridization (ISH) and immunohistochemistry (IHC) are summarized on a 4-point scale (Table), although the techniques used provide only semiquantitative data.

ECE Extraction and Activity

Membranes were prepared at 4°C from 80 mg of APA.[23] Big ET-1 was converted to ET-1 by incubation of the solubilized membranes (50 to 200 μg protein) with 0.1 μmol/L big ET-1 for 30 minutes at 37°C. The specificity of the ECE enzymatic activity was checked with phosphoramidon, a zinc metalloprotease inhibitor. The mixture was assayed using a commercial enzyme immunoassay kit (Cayman Chemical Co) to quantify ET-1.

Results

ET System in APAs

Tissue samples from 20 patients of the COMETE network were analyzed, and the results shown are representative of all the cases. Histological examination of the specimens revealed a cortical adenoma in all cases; the appearance was heterogeneous, with a predominant hyperplasic zona glomerulosa (not shown).

RT-PCR of frozen tissue gave strong amplification of ECE-1 in all samples, low amplification of ET_A and ET_B mRNAs, and little or no amplification of PPET-1 (Figure 1).

The distribution of each component of the ET system in adrenocortical adenomas was determined by ISH. The results were in agreement with the RT-PCR data. Figure 2 is representative of the 20 APA samples studied. Dark-field and bright-field microphotographs of labeling with each riboprobe showed diffuse ET_A receptor mRNA (Figure 2a and 2b), a high concentration of ET_B receptor mRNA mainly on the framework of the secretory cells (Figure 2c and 2d), clumps of intense ECE-1 mRNA hybridization (Figure 2e and 2f), and no specific signal for PPET-1 mRNA (Figure 2g and 2h). ET_A mRNA labeling was stronger in the compact cells than in the lipid-rich cells (Figure 2b), whereas ECE-1

Cellular Distribution of the Endothelin Components

			PPET-1		ECE-1	
Cell Type	ET_A mRNA	ET_B mRNA	mRNA	Protein	mRNA	Protein
Secretory	+	−	−	−	+++	+++
Endothelial	s	++	−*	+	++	+
Smooth muscle	+	++	−	+	+	+

For each endothelin component, − indicates no labeling; +, low labeling; ++, moderate labeling; +++, high labeling in the vast majority of cells in the designated population; *, labeling only in scattered cells; and †, no labeling except in the 6 samples in which thickened precapillary arterioles were found.

Amounts of mRNA were evaluated from ISH studies and amounts of proteins from immunohistochemistry. The average values are representative of the 20 specimens examined and are consistent among specimens when comparisons were carried out separately for each probe.

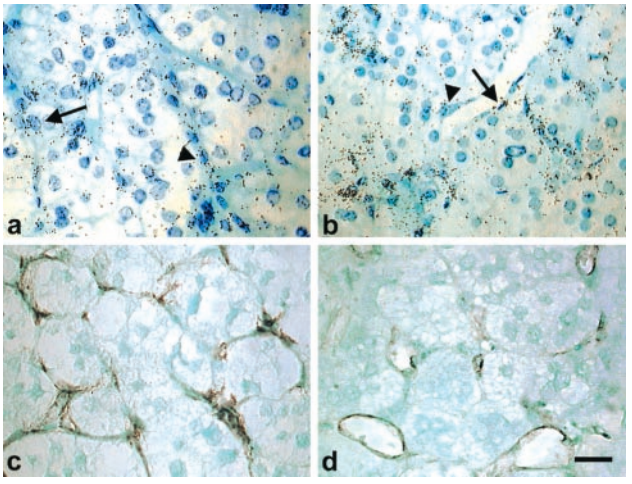

Figure 1. Components of the ET system in APAs. ET_A and ET_B receptors, ECE-1, PPET-1, and GAPDH mRNAs were detected by RT-PCR using the specific primers described previously[22] and total RNA from the 20 frozen adrenal cortex samples. Only 5 samples are shown in Figure 2. Thirty cycles of amplification gave each gene at the expected size: 675 bp (ET_A), 400 bp (ET_B), 341 bp (PPET-1), 622 bp (ECE-1), and 649 bp (GAPDH). The molecular weight markers shown on the right are λ/*Hin*dIII, ϕX174 DNA/*Hae*III.

mRNA was concentrated in glomerular lipid-rich cells but sparse in the adjacent compact zona glomerulosa–like cells (Figure 2f). ET_B mRNA labeling was intense in the zona glomerulosa but weak in the zona fasciculata and mainly confined to capillaries running among tumor cells (Figure 2d). Sense probes yielded no labeling in any tissue (not shown).

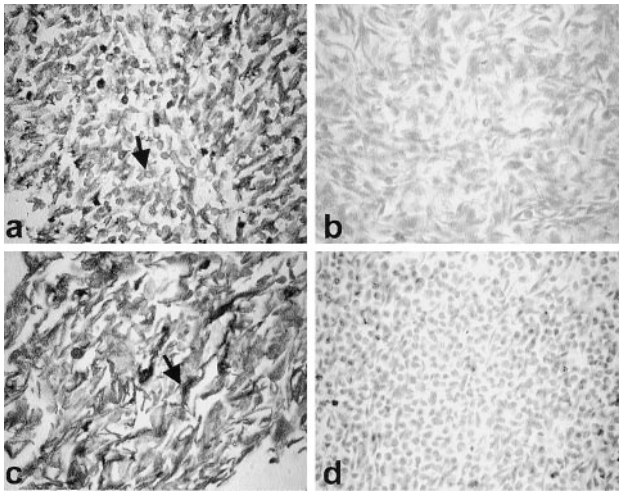

Figure 2. Localization of the ET system in APAs. ISH was performed on sections of adrenal cortex from the 20 patients with the antisense probes for ET_A (a and b), ET_B (c and d), ECE-1 (e and f), and PPET-1 (g and h). Photographs representative of the 20 samples studied are shown in dark-field illumination (left) and in bright-field illumination (right). ET_A mRNA was found in compact cells of the zona glomerulosa (arrow, a) and ET_B mRNA in vascular structures (arrow, c). ECE-1 mRNA labeling was ubiquitous and intense, and no specific signal for PPET-1 mRNA was detected. Scale bar 50 μm.

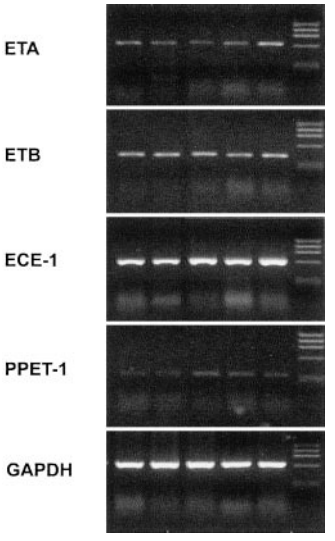

Figure 3. Cellular distribution of ET_A and ET_B receptor mRNAs in APAs. ISH with the antisense probe for ET_A (a) and ET_B (b) receptors. Microphotographs representative of the 20 samples studied are shown in bright-field illumination. Immunohistochemistry was performed with anti-αSMA (c) (smooth muscle cell marker) and anti-CD31 (d) (endothelial cell marker) antibodies to identify the cells labeled with the ET_A and ET_B probes. ET_A mRNA labeling was found in secretory cells (arrow) and smooth muscle cells (arrowhead), and ET_B mRNA labeling was found mainly in endothelial cells (arrow) and smooth muscle cells (arrowhead). Scale bar 20 μm.

Cellular Distribution of the ET System in APAs

Figure 3 shows the cellular distribution of receptor transcripts in APAs. Semiquantitative evaluation was performed on the 20 samples (Table). ET_A mRNA (Figure 3a) was found in secretory cells (score, +), in the smooth muscle layer of arteries, and in a few smooth muscle cells (score, +) surrounding arterioles that stained for α-SMA (Figure 3c). In contrast, ET_B mRNA (Figure 3b) was found mainly within the network of capillary sinusoids, with high concentrations in endothelial cells (score, ++) that stained for CD-31 (Figure 3d) and in α-SMA–positive cells adjacent to the vessels (score, ++) (Figure 3c). Secretory cells were occasionally labeled.

We tested a specific anti–ET-1 antibody. The ET-1 antibody labeled CHO/PPET-1/ECE-1 cells producing ET-1 (Figure 4a) and CHO/ET_B cells incubated with ET-1 (Figure 4c) under the same conditions as those used for the APA sections, but not CHO/ET_B cells alone (Figure 4b). The antibody did not react nonspecifically with untransfected CHO cells (Figure 4d). Thus, the antibody labeled cells synthesizing ET-1 as well as cells binding ET-1.

Figure 5 shows the cellular distribution of ECE-1 (Figure 5a and 5b) and PPET-1 (Figure 5c to 5f). There was a strong signal for ECE-1 mRNA in several cell types, mainly secretory cells (score, +++) (Figure 5a) and some endothelial cells (score, ++) (Table). ECE-1 immunoreactivity correlated perfectly with the ISH results, confirming the high concentration of the enzyme (Figure 5b). No signal for PPET1 mRNA was found in any structure (Figure 5c). However, ET-1 immunoreactivity was found in endothelial cells (score, ++) (Figure 5d), showing a discrepancy be-

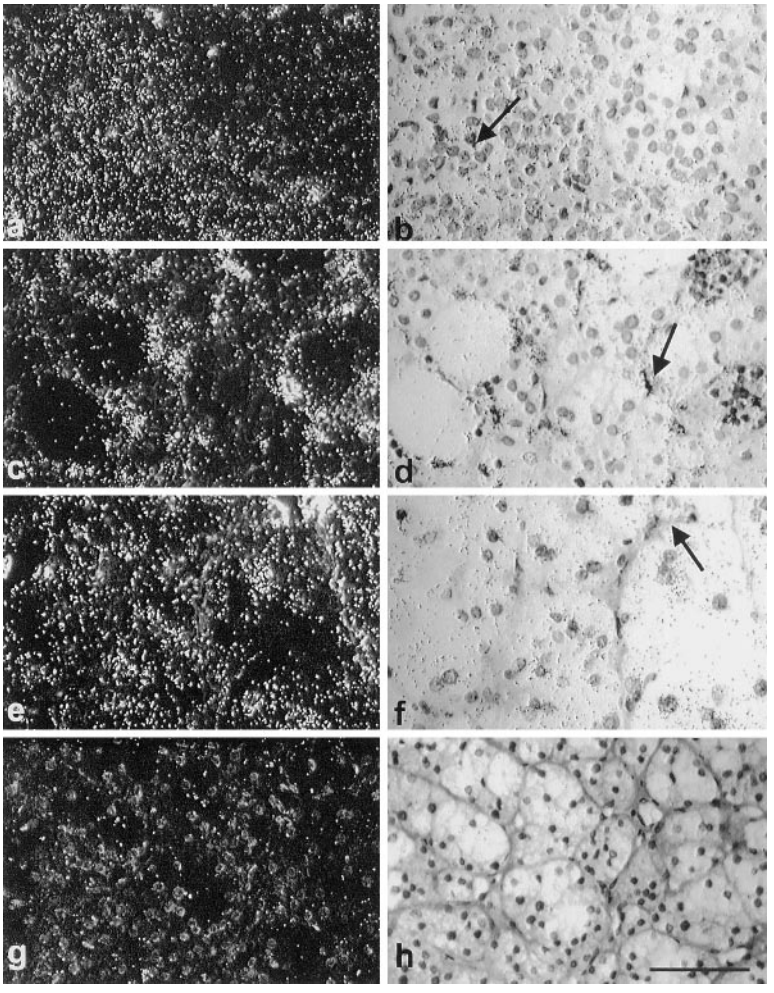

Figure 4. ET-1 immunoreactivity. ET-1 immunohistochemistry was performed in paraffin-embedded specimens of stable transfected CHO/PPET-1/ECE-1 cells (a), CHO/ET_B cells (b), CHO/ET_B cells incubated with ET-1 for 30 minutes at 4°C (c), and untransfected CHO cells (d). CHO/PPET-1/ECE-1 cells contained ET-1 that was recognized by the antibody (arrow, a). No immunoreactivity was detected in CHO/ET_B cells (b) or in untransfected CHO cells (d). In contrast, ET-1 incubated with CHO/ET_B and bound to ET_B receptors was recognized by the anti–ET-1 antibody (arrrow, c).

tween the de novo synthesis of PPET-1 and the presence of ET-1 peptide.

Only 6 samples of APA adrenal cortex presented a strong labeling for PPET-1 mRNA (Figure 5e), which localized to the thickened wall of some precapillary arterioles characterized by Lack et al.[24] The high concentration of PPET-1 mRNA was correlated with ET-1 immunoreactivity (Figure 5f). The thickened capillary arterioles also had ECE-1 in their endothelial and perivascular cells and ET_A in the smooth muscle cells (not shown).

ECE-1 Activity in the APA Adrenal Cortex

Solubilized membranes were prepared from a sample of APA adrenal cortex, and the enzymatic properties of ECE-1 were measured. The enzyme activity converted 0.1 μmol/L big ET-1 at a rate of 3.7 pg $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$ and was >90% inhibited by phosphoramidon.

Discussion

We examined the role of the ET system in endocrine glands. Patients with Conn's syndromes were studied because their tumors arise from the zona glomerulosa of the adrenal cortex. The high concentrations of human ECE-1 mRNA and its protein in the adrenal gland[11] suggest that ECE-1 is involved in other systems, such as the regulation of hormone secretion, rather than simply generating ET-1 from its precursor.

We tested this notion by determining the amounts and the cell-specific distributions of the ET system in human APAs. The adrenal cortex of APAs contains high concentrations of ECE-1 and ET receptor mRNAs, but PPET-1 mRNA is not easily detected. The Table summarizes the cellular localization of the components of the ET system, showing that secretory cells contain ET_A mRNA and ECE-1 mRNA and protein, whereas the vascular structures contain both receptors, ECE-1 mRNA and protein, as well as ET-1 peptide probably associated with the receptors. To our knowledge, this is the first report of the cellular distributions of PPET-1, ET_A, and ET_B mRNAs in the human adrenal.

The distribution of ET receptor mRNAs determined by ISH is in good agreement with previous results of autoradiography that could not elucidate the type of cells showing the binding.[14] ET-1 acts as secretagogue for calf adrenal zona glomerulosa cells[25] and is trophic for rat adrenal zona glomerulosa cells.[26] These authors suggested that ET-1 could be the factor that causes enhanced aldosterone secretion in APAs. The decrease in ET_B receptors reported by Rossi et al[27] and Zeng et al[17] could cause the higher plasma ET-1 concentrations described by some,[16] as ET_B may also function as a physiological clearance receptor for extracellular ET-1.[28] Lariviere et al[29] suggested that the increased ET-1 content is due to its involvement in the maintenance of high blood

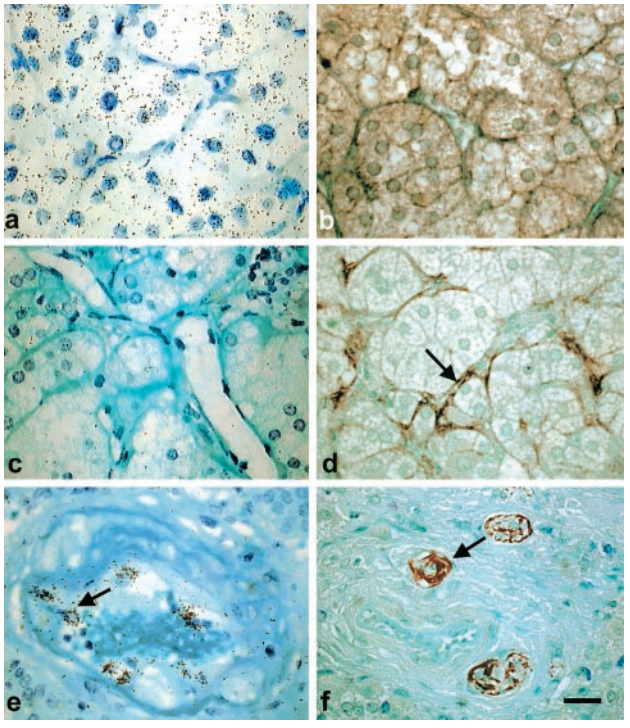

Figure 5. Cellular distribution of PPET-1 and ECE-1 mRNAs in APAs. ISH with the antisense probes for ECE-1 (a) and PPET-1 (c and e). Microphotographs representative of the 20 APA samples studied are shown in bright-field illumination. Immunohistochemistry was performed with the anti–ECE-1 (b) and anti–ET-1 (d and f) antibodies. There was intense ubiquitous labeling for ECE-1 mRNA and protein, whereas no specific labeling was detected for PPET-1 mRNA (c). However, strong ET-1 immunoreactivity was found in endothelial cells (arrow, d). Thickened precapillary arterioles were found in 6 of the 20 samples and contained PPET-1 mRNA (arrow, e) and ET-1 (arrow, f). Scale bar 20 μm.

pressure in mineralocorticoid hypertension. The vascular distribution of both ET receptors, plus the presence of ET_A in some secretory cells, supports the proposal of Hinson et al[10] that ET-1 regulates aldosterone secretion in the adrenal glands in a paracrine fashion.

ET-1 is involved in adrenal steroid secretion, but its cellular origin remains unclear. RT-PCR of whole APA cortex resulted in a faint band for PPET-1, in accordance with published reports,[9,27] although PPET-1 mRNA has been detected in dispersed and purified human adrenocortical cells.[30,31] However, we detected no PPET-1 signal on secretory cells containing ECE-1 by ISH. One possible explanation is that the low level of transcription is not detected by ISH. Otherwise, RT-PCR amplified PPET-1 mRNA from a contaminant vessel of the capsule, or the technique used to disperse adrenocortical cells activated the transcription of PPET-1. Immunohistochemical studies are somewhat inconsistent about the distribution of ET-1 immunoreactivity. Some reports are in agreement with the present report, with ET-1 immunoreactivity confined to endothelial cells.[12] Others found it in parenchymal cells,[32,13] although it was recently demonstrated that the immunoreactive protein is ET-1–like and is a different size than PPET-1 or ET-1.[33]

Our experiments with cell lines showed that the anti–ET-1 antibody we used recognized both endogenous ET-1 and ET-1 bound to its receptor, suggesting that the discrepancy between ISH and IHC is due to the detection of ET-1 bound to ET_B. A recent report showed that internalized ET-1–stimulated ET_A receptors are rapidly recycled to the membrane, whereas internalized ET_B receptors with almost irreversibly bound ET-1 are degraded more slowly.[34] This would explain why receptor-bound ET-1 immunoreactivity was seen only on ET_B-labeled cells and not in ET_A receptors.

The presence of PPET-1 in the thickening of the walls of the precapillary arterioles of patients with a lymphocytic vasculariti[24] is not surprising because this gene is very sensitive to shear stress and hypoxia and its transcription is enhanced in the majority of vascular diseases (see Miyauchi and Masaki[2] for a recent review). The presence of ECE-1 in secretory cells in APAs might not be a hallmark of ET-1 biosynthesis but indicates an alternative function of ECE-1. Johnson et al[35] recently showed that ECE-1 has a broad enzymatic activity for substrates other than big ETs, some of which are present in the adrenal cortex.

We conclude that ET-1 is not synthesized de novo in the human adrenal. The presence of ET within endothelial cells of adrenal capillaries could also indicate very low levels of newly synthesized ET-1. This would be consistent with a paracrine-autocrine role for ET-1 in controlling adrenal blood flow, as proposed by Davenport et al.[12] However, several questions remain unanswered, such as the function of the surprisingly large amount of ECE-1.

Acknowledgments

We thank Prof P.-F. Plouin and the COMETE network, and we thank Dr C. Parnot for providing CHO/PPET-1/ECE-1 cells. Dr Egidy was supported by funding from “La Fondation pour la Recherche Médicale.” The English text was edited by Dr Owen Parkes.

References

1. Nussdorfer G, Rossi GP, Malendowicz LK, Mazzochi G. Autocrine-paracrine endothelin system in the physiology and pathology of steroid-secreting tissues. *Pharmacol Rev*. 1999;51:403–437.
2. Miyauchi T, Masaki T. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol*. 1999;61:391–415.
3. Hosoda K, Nakao K, Hiroshi S, Suga S, Ogawa Y, Mukoyama M, Shirami G, Saito Y, Nakanishi S, Imura H. Cloning and expression of human endothelin-1 receptor cDNA. *FEBS Lett*. 1991;287:23–26.
4. Sakamoto A, Yanagisawa T, Sakurai T, Takuwa Y, Yanagisawa H, Masaki T. Cloning and functional expression of human cDNA for the ET_B endothelin receptor. *Biochem Biophys Res Commun*. 1991;178:656–663.
5. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell*. 1994;78:473–485.
6. Valdenaire O, Rohrbacher E, Mattei M. Organization of the gene encoding the human endothelin-converting enzyme (ECE-1). *J Biol Chem*. 1995;270:29794–29798.
7. Emoto N, Yanagisawa M. Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J Biol Chem*. 1995;270:15262–15268.
8. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayishi Y, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent, vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988;332:411–415.
9. Imai T, Hirata Y, Eguchi S, Kanno K, Ohta K, Emori T, Sakamoto A, Yanagisawa M, Masaki T, Marumo F. Concomitant expression of receptor subtype and isopeptide of endothelin by human adrenal gland. *Biochem Biophys Res Commun*. 1992;182:1115–1121.
10. Hinson J, Vinson G, Kapas S, Teja R. The role of endothelin in the control of adrenocortical function: stimulation of endothelin release by ACTH and the effects of endothelin-1 and endothelin-3 on steroido-

genesis in rat and human adrenocortical cells. *J Endocrinol*. 1991;128: 275–280.
11. Korth P, Bohle R, Corvol P, Pinet F. Cellular distribution of endothelin-converting enzyme-1 in human tissues. *J Hystochem Cytochem*. 1999;47: 447–462.
12. Davenport A, Hoskins S, Kuc R, Plumpton C. Differential distribution of endothelin peptides and receptors in human adrenal gland. *Histochem J*. 1996;28:779–789.
13. Hiraki H, Hoshi N, Hasegawa H, Tanigawa T, Emura I, Seito T, Yamaki T, Fukuda T, Watanabe K, Suzuki T. Regular immunohistochemical localization of endothelin-1 and endothelin-B receptor in normal, hyperplasic and neoplastic human adrenocortical cells. *Pathol Int*. 1997;47: 117–125.
14. Rossi G, Albertin G, Belloni A, Zanin L, Biasolo M, Prayer-Galeti T, Bader M, Nussdorfer G, Palu G, Pessina A. Gene expression, localization, and characterization of endothelin A and B receptors in the human adrenal cortex. *J Clin Invest*. 1994;94:1226–1234.
15. Vierhapper H, Nowotny P, Waldhausl W. Effect of endothelin-1 in man: impact on basal and adrenocorticotropin-stimulated concentrations of aldosterone. *J Clin Endocrinol Metab*. 1995;80:948–951.
16. Letizia C, DeToma G, Cerci S, Scuro L, DeCiocchis A, D'Ambrosio C, Massa R, Cavallaro A, Scavo D. Plasma endothelin-1 levels in patients with aldosterone-producing adenoma and pheochromocytoma. *Clin Exp Hypertens*. 1996;18:921–931.
17. Zeng Z, Tang X, Yang D, Li H, Zhang R, Zeng Q, Li M, Chen J, Lu Z, Demura H, Naruse M, Shi Y. Immunoreactive endothelin-1 and its receptors in human adrenal tissues. *J Cardiovasc Pharmacol*. 1998;31: S212–S214.
18. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156–159.
19. Shimada K, Matsushita Y, Wakabayashi K, Takahashi M, Matsubar A, Iijima Y, Tanzawa K. Cloning and functional expression of human endothelin-converting enzyme cDNA. *Biochem Biophys Res Commun*. 1995;207:807–812.
20. Itoh Y, Yanagisawa M, Ohkubo S, Kimura C, Tosaka T, Inoue A, Ishida N, Mitsui Y, Onda H, Fujino M. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett*. 1988;231:440–444.
21. Tso J, Sun X, Kao T, Reece K, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucl Acids Res*. 1985;13:2485–2502.
22. Egidy G, Juillerat-Jeanneret L, Korth P, Bosman F, Pinet F. The endothelin system in human normal colon. *Am J Physiol*. 2000;279: G211–G222.
23. Korth P, Egidy G, Parnot C, LeMoullec J, Corvol P, Pinet F. Construction, expression and characterization of a soluble form of human endothelin-converting enzyme. *FEBS Lett*. 1997;417:365–370.
24. Lack EE. *Tumors of the Adrenal Gland and Extra-Adrenal Paraganglia: Atlas of Tumor Pathology*. Washington, DC: Armed Forces Institute of Pathology; 1997;19:91–122.
25. Cozza E, Gomez-Sanchez C, Foecking M, Chiou S. Endothelin binding to cultured calf adrenal zona glomerulosa cells and stimulation of aldosterone secretion. *J Clin Invest*. 1989;84:1032–1035.
26. Mazzochi G, Rossi G, Rebuffat P, Malendowicz L, Markowska A, Nussdorfer G. Endothelins stimulate deoxyribonucleic acid synthesis and cell proliferation in rat adrenal zona glomerulosa, acting through an endothelin A receptor coupled with protein kinase C- and tyrosine kinase-dependent signaling pathways. *Endocrinology*. 1997;138:2333–2337.
27. Rossi G, Belloni A, Albertin G, Zanin L, Biasolo M, Nussdorfer G, Palu G, Pessina A. Endothelin-1 and its receptors A and B in human aldosterone-producing adenomas. *Hypertension*. 1995;25:842–847.
28. Dupuis J, Stewart D, Cernacek P, Gosselin G. Human pulmonary circulation is an important site for both clearance and production of endothelin-1. *Circulation*. 1996;94:1578–1584.
29. Lariviere R, Thibault G, Schiffrin E. Increased endothelin-1 content in blood vessels of deoxycorticosterone acetate-salt hypertensive but not in spontaneously hypertensive rats. *Hypertension*. 1993;21:294–300.
30. Rossi G, Albertin G, Neri G, Andreis P, Hofmann S, Pessina A, Nussdorfer G. Endothelin-1 stimulates steroid secretion of human adrenocortical cells ex vivo via both ET_A and ET_B receptor subtypes. *J Clin Endocrinol Metab*. 1997;82:3445–3449.
31. Mathieu M, Hamroun D, Pouzeratte Y, Guerineau N, Guillon G, Chevillard C. Adrenal steroid-secreting cells express endothelin-1. *Endocrin Res*. 1998;24:743–747.
32. Li Q, Grimelius L, Gröndal S, Höög A, Johansson H. Immunohistochemical localization of endothelin-1 in non-neoplasic adrenal gland tissue. *Virchows Arch Pathol Anat*. 1994;425:259–264.
33. Li Q, Grimelius L, Höög A, Johansson H, Kjellman M, Larsson A. Characterization of endothelin-1-related protein in human adrenal cortex and in cortical lesions. *Histochem Cell Biol*. 1999;111:33–38.
34. Bremnes T, Paasche J, Mehlum A, Sandberg C, Bremnes B, Attramadal H. Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem*. 2000;275:17596–17604.
35. Johnson G, Stevenson T, Ahn K. Hydrolysis of peptide hormones by endothelin-converting enzyme-1: a comparison with neprilysin. *J Biol Chem*. 1999;274:4053–4058.