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Autocrine-Paracrine Role of Endothelin-1 in the Regulation of Aldosterone Synthase Expression and Intracellular Ca^{2+} in Human Adrenocortical Carcinoma NCI-H295 Cells*

GIAN PAOLO ROSSI, GIOVANNA ALBERTIN, SERGIO BOVA, ANNA S. BELLONI, FRANCESCO FALLO, UBERTO PAGOTTO, LUCIA TREVISI, GIORGIO PALÙ, ACHILLE C. PESSINA, AND GASTONE G. NUSSDORFER

Departments of Clinical and Experimental Medicine, Pharmacology (S.B., L.T.), Division of Endocrinology (F.F.), Institutes of Microbiology (G.P.) and Anatomy (A.S.B., G.G.N.), University of Padua Medical School, Padova, Italy; and Max Planck Institute of Psychiatry, Clinical Institute (U.P.), Munich, Germany

ABSTRACT

The role played by endothelin (ET-1) and its receptor subtypes A and B (ET_A and ET_B) in the functional regulation of human NCI-H295 adrenocortical carcinoma cells has been investigated. Reverse transcription-PCR with primers specific for prepro-ET-1, human ET-1 converting enzyme-1, ET_A, and ET_B complementary DNAs consistently demonstrated the expression of all genes in NCI-H295 cells. The presence of mature ET-1 and both its receptor subtypes was confirmed by immunocytochemistry and autoradiography, respectively. Aldosterone synthase (AS) messenger RNA was also detected in NCI-H295 cells, and AS gene expression was enhanced by both ET-1 and the specific ET_B agonist IRL-1620; this effect was not inhibited by either the ET_A antagonist BQ-123 or the ET_B antagonist BQ-788. A clear-cut increase in the intracellular Ca^{2+} concentration in NCI-H295 cells in response to ET_B, but not ET_A, activation was observed. In light of these findings, the following conclusions can be drawn: 1) NCI-H295 cells possess an active ET-1 biosynthetic pathway and are provided with ET_A and ET_B receptors; 2) ET-1 regulates in an autocrine/paracrine fashion the secretion of aldosterone by NCI-H295 cells by enhancing both AS transcription and raising the intracellular Ca^{2+} concentration; and 3) the former effect of ET-1 probably involves the activation of both receptor subtypes, whereas calcium response is exclusively mediated by the ET_B receptor. (*Endocrinology* **138:** 4421–4426, 1997)

ENDOTHELIN-1 (ET-1), the prototype of a family of 21-amino acid residue peptide hormones (1), exerts multiple biological effects, including very potent vasoconstriction, mitogenesis, stimulation of protooncogene expression, inhibition of renin secretion, and stimulation of catecholamines, vasopressin, and aldosterone secretion (for review, see Refs. 2 and 3). The latter effect was observed both *in vivo* in animals and *in vitro*, and is potentially important in conditions where enhanced ET-1 and aldosterone secretions coexist, such as severe and/or malignant hypertension, congestive heart failure, and hypoxia (for review, see Ref. 4). We recently demonstrated the expression of the genes for prepro-ET-1 and of its receptors A and B (ET_A and ET_B) in homogenates of the normal adrenal cortex of rats and humans as well as in aldosterone-producing tumors (5–7) and showed that the mineralocorticoid secretagogue effect of ET-1 is mediated in the rat by the ET_B receptor (6; for review, see Ref. 8). However, whether this also applies to human adrenocortical cells is not known at present.

A human adrenocortical tumor cell line (NCI-H295) is now available (9) that expresses angiotensin II AT1 receptor functionally coupled to phosphoinositidase C, secretes aldosterone in response to angiotensin II, and has been widely used for investigating the regulation of adrenal steroidogenesis *in vitro* (10–13). Hence, we hypothesized that NCI-H295 cells can be useful for investigating the role of ET-1 in the regulation of human adrenocortical function.

This study was, therefore, designed to investigate whether NCI-H295 cells 1) express the genes for prepro-ET-1, human ET-1 converting enzyme-1 (hECE-1), and ET_A and ET_B receptors; 2) are endowed with a functional ET-1 biosynthetic pathway; 3) possess functional ET_A and ET_B receptor subtypes; and 4) regulate the expression of the aldosterone synthase (AS) gene in response to ET-1.

Materials and Methods

Reagents

Unless otherwise stated, laboratory reagents were obtained from Sigma Chemical Co. (Milan, Italy). ET-1 was obtained from either Peninsula Laboratories (Merseyside, UK) or Neosystem Laboratories (Strasbourg, France). The ET agonists and antagonists used were BQ-123, a selective ET_A antagonist (14); BQ-788, a potent and selective ET_B antagonist (15); sarafotoxin 6C, a weak ET_B agonist (16); and IRL-1620, a potent ET_B agonist (17). They were purchased from Neosystem Labo-

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Address all correspondence and requests for reprints to: Gian Paolo Rossi, M.D., F.A.C.C., Clinica Medica 1, Dipartimento di Medicina Clinica e Sperimentale, University Hospital via Giustiniani, 2, 35126 Padova, Italy. E-mail: gprossi@ipdunidx.unipd.it.
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ratories. [^{125}I]ET-1 (SA, 2000 Ci/mmol) was purchased from Amersham Laboratories (Aylesbury, UK).

Cell culture

Human NCI-H295 adrenal carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD; catalogue no. CRL 10296). Cells were initially maintained in RPMI 1640 medium (Life Technologies, Eggenstein, Germany; catalogue no. 041–02404M) supplemented with 2% FCS, insulin (0.005 mg/ml; Monotard, Novo Nordisk Farmaceutici, Rome, Italy), transferrin (0.01 mg/ml), sodium selenite (30 nM), and antibiotics (penicillin-streptomycin). Cells were maintained and grown on 25-cm^2 flasks at 37 C under an atmosphere of 5% CO_2-95% air. During the initial 2 months of culture, only attached cells were retained when medium was changed. Cells used for the experiments described were routinely maintained as monolayer cultures. When the responses of cells to agonists were studied, the medium was removed and replaced with serum-free medium and 0.02% BSA.

NCI-H295 cells were used for RNA extraction by the guanidium isothiocyanate method. RNA was precipitated by the addition of 500 μl ethanol and 20 μl 3 M sodium acetate and standing for 1 h at −80 C. It was recovered by centrifugation (15 min, 12,000 × *g*, 4 C), and the pellet was washed once in 75% ethanol (1.0 ml) before being dried under vacuum and dissolved in diethylpyrocarbonate-water. After isolation, total RNA was checked for integrity by 1.5% agarose gel electrophoresis and quantified by measurement of UV absorbance at 260 nm.

Reverse transcription-PCR (RT-PCR)

For use in the PCR, total RNA was reversely transcribed to complementary DNA (cDNA) with random hexamers (2.5 μM), as previously detailed (5). After incubation at 42 C for 15 min, temperature was raised to 95 C for 5 min and then quickly decreased to 5 C for 5 min. For amplification of the resulting cDNA, 20 μl of the reverse transcription mixture were used. The sample volume was increased to 100 μl with a solution containing 50 mM KCl, 10 mM Tris (pH 8.3), 2 mM $MgCl_2$, and 0.2 μM of up- and downstream primers as well as 2.5 U *Taq* polymerase (AmpliTaq DNA Polymerase, Perkin-Elmer/Cetus, Norwalk, CT). Amplification of prepro-ET-1, ET_A, ET_B, and hECE-1 cDNA was carried out with the primers and the thermal profiles previously reported (5, 18), using a Delphi 1000 Thermal Cycler (MJ Research, Waterston, MA). The specificity of the amplification products for the gene of interest was confirmed by hybridization with the following cDNA-specific probes: hECE-1, 5′-TTG GAC TTT GAG ACG GCA CTG GC-3′; ET_A receptor, 5′-CCT CAA CCT CTG CGC TCT TAG TGT-3′; and ET_B receptor, 5′-TCC TGC CTT GTG TTC GTG CTG GGG-3′. As a positive control, amplification of a 838-bp fragment of the human β-actin gene was carried out in parallel, as described previously (5). As false positive results of RT-PCR for the β-actin gene, due to amplification of retropseudogenes, have been reported (19), in parallel experiments ET_A and ET_B receptor PCR was carried out with no prior RT to further rule out the possibility of amplifying genomic DNA.

Immunocytochemistry

NCI-H295 cells were cultured in double slide flasks at a concentration of 20,000 cells/well in RPMI 1640 with all ingredients and 2% FCS for 4 days, then washed in PBS and cultured for 24 h in RPMI supplemented only with antibiotics, glutamine, selenium, and transferrin. Immunocytochemistry was performed with minor modifications as previously described (20). Briefly, two antibodies for ET-1 were used: a rabbit polyclonal (Peninsula, Heidelberg, Germany) and a mouse monoclonal (Dianova, Hamburg, Germany; cross-reactivity for ET-3, 7% and 2%, respectively). Rabbit and swine serum and biotinylated swine antirabbit and antimouse IgGs were obtained from Dako (Hamburg, Germany). After medium removal and several washes in cold PBS, the cell monolayer was rapidly dried and fixed in 4% paraformaldehyde, and endogenous peroxidase was blocked with 1% H_2O_2. The sections were then preincubated in rabbit or swine serum (1:10), followed by application of polyclonal or monoclonal ET-1 antibody (1:1500 and 1:125) overnight at 4 C. After incubation with the respective biotinylated IgGs (1:300) and the streptavidin-biotin-horseradish peroxidase complex (1:100) for 1 h at room temperature each, the enzymatic reaction was performed for 5–10 min at room temperature using 0.1% 3,3-diaminobenzidine tetrahydrochloride (diluted in PBS with 0.05% hydrogen peroxide) as chromogen. After immunostaining, the sections were inserted for dehydration in a series with increasing concentrations of ethanol-xylol, and the coverslips were attached with Eukitt (Riedel de Haen, Seelze, Germany). For immunocytochemical detection, negative controls with omission of the primary antibody were run in parallel.

Autoradiography

Cell monolayers were immediately frozen at −30 C by immersion in isopentane and stored at −80 C. They were processed according to the method of Kuhar (21) with minor modifications (6). ET-1-binding sites were labeled *in vitro* by incubation for 120 min with 100 pM [^{125}I]ET-1; nonspecific binding was determined by adding 1 μM unlabeled ET-1. Selective [^{125}I]ET-1 binding to ET_A and ET_B was studied by adding 100 nM BQ-123 and sarafotoxin 6C, respectively. The reaction was stopped by washing the samples three times in 50 nM Tris-HCl buffer. After rinsing, the sections were rapidly dried, fixed in paraformaldehyde vapors at 80 C for 120 min, and coated with NTB2 Kodak nuclear emulsion (Eastman Kodak, Rochester, NY). The autoradiograms were exposed for 2 weeks at 4 C and then developed with undiluted D19 Kodak developer. They were stained with hematoxylin-eosin, and observed and photographed with a Leitz Laborlux microscope (Leitz, Rockleigh, NJ). For the purpose of quantitation, autoradiograms were analyzed by computer-assisted densitometry using a camera-connected microscope and a personal computer equipped with software specifically written for this purpose (Studio Casti Imaging, Venice, Italy). Given the uneven distribution of cells on monolayers, only areas corresponding to identifiable cells were outlined and measured. For each autoradiogram (n = 4), 10–18 areas of about 55,000 μm^2 (79,500 pixels) were analyzed. The density of the ET_A subtype was assessed in the presence of the ET_B weak agonist sarafotoxin 6C (500 nM), and that of ET_B was determined in the presence of BQ-123 (500 nM).

Gene expression

NCI-H295 cells (5×10^5 cells/well) were incubated for 24 h at 37 C with ET-1 (10^{-8} M) or IRL-1620 (10^{-7} M) and with ET-1 (10^{-8} M) plus BQ-123 or BQ-788 (10^{-6} M). At the end of the incubation period, cells were harvested, and RNA was extracted and reversed transcribed (see above). PCR amplification was performed (see above) with 21-mer 5′-ACATTGGTACAGGTTTTCCTC-3′ (sense) and 5′-CAGATGCAAGACTAGTTAATC-3′ (antisense) (22). To evaluate the kinetics of the amplification reaction, 5-μl aliquots of the amplification mixture were collected after every two cycles starting from the 20th up to the 38th cycle. Aliquots of the PCR products were transferred to a nylon membrane (Immobilon-S, Millipore, Milan, Italy) by a slot blot apparatus (Milliblot, Millipore) and UV cross-linked (Stratagene UV-Crosslinker 1800, Stratagene-Duotech, Milan, Italy). Detection of the digoxigenin-labeled amplification products on the nylon membrane was carried out by high affinity antidigoxigenin antibody Fab fragments conjugated to alkaline phosphatase using a chemiluminescent detection kit (DIG Luminescent Detection Kit, Boehringer Mannheim, Mannheim, Germany). Light generated via dephosphorylation of the chemiluminescent substrate (CSPD, Boehringer Mannheim) was used to impress x-ray films (BioMax MR-1 film, Sigma) with a 20-min exposure, as in an autoradiographic procedure (5). Quantification of the PCR products was carried out by measuring the integrated optical density of the autoradiographies with an image analyzer IBAS 2000 (Zeiss, Unterkochen, Germany). Plots of the integrated optical density *vs.* the number of cycles were then elaborated; the cycle number (N_{50}) that corresponded to the half-maximal PCR products was also calculated as an estimate of the amount of initial amplifiable template of the gene (5, 23). Comparison of the N_{50} of cells exposed to the different agonists and antagonists was carried out by one-way ANOVA followed by Bonferroni *post-hoc* test; the significance level was set at 0.05.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured by fluorometry in a double wavelength mode in a Perkin-Elmer LS-50B spectrofluorometer equipped with a magnetic

stirrer and a temperature regulator (Perkin-Elmer, Norwalk, CT). Cell monolayers were loaded with fura-2/acetoxymethyl ester (fura-2/AM; Molecular Probes, Eugene, OR) as described by Bird *et al.* (24). Briefly, NCI-H295 cells were plated on 8 × 30-mm glass coverslips and cultured in growth medium, as described above, for 7 days. For cell loading, coverslips were incubated in a buffer (130 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 15 mM glucose, 1 mg/ml BSA, and 10 mM HEPES, pH 7.4) containing 5 μM fura-2/AM for 45 min at 37 C in a 5% CO_2-95% O_2 atmosphere. Coverslips were then transferred to a customized holder, inserted into a quartz cuvette, and placed into the spectrofluorometer, where they were superfused at a rate of 5 ml/min with the same buffer without BSA (25). The cells were left to recover for 20 min before starting the experiment. $[Ca^{2+}]_i$ was calculated by the formula of Grynkiewicz *et al.* (26): $[Ca^{2+}]_i = \{[(R - R_{min})/(R_{max} - R)] \times (S_f/S_b) \times K_d\}$, where K_d is the dissociation constant of fura-2 and was assumed to be 225 nM, R is the 340/380 nm ratio of fura-2 fluorescence measured in the cells, R_{max} is the 340/380 nm ratio in the presence of a saturating calcium concentration, R_{min} is the 340/380 nm ratio of fura-2 fluorescence in a Ca^{2+}-free solution containing EGTA, and S_f/S_b is the ratio of Ca^{2+}-free to Ca^{2+}-bound fura-2 fluorescence at 380 nm. Calibration was performed by using fura free acid. R_{min} was determined in a solution containing 115 mM KCl, 10 mM NaCl, 2 mM $MgSO_4$, 10 mM K_2H_2-EGTA, and 10 mM K_2-3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.2. For the determination of R_{max}, 2 mM $CaCl_2$ was added to the medium, and CaK_2-EGTA was substituted for K_2H_2-EGTA. R_{max}, R_{min}, and S_f/S_b values were 17.36, 1, and 7.81, respectively.

The effect of ET-1 (10 nM) and the ET_B agonist IRL 1620 (100 nM) on $[Ca^{2+}]_i$ was assessed with and without 10-min pretreatment with BQ-788 (0.8 mM) and/or BQ-123 (1 mM). As exposure to ET-1 was reported to induce homologous down-regulation (27), each experiment was carried out on a different preparation of NCI-H295 cells. Each set of experiments was carried out four times on different cell preparations with consistent results.

Results

Analysis of prepro-ET-1, ET_A, ET_B, and hECE-1 messenger RNA (mRNA) levels

RT-PCR analysis of RNA from NCI-H295 cells showed prepro-ET-1, hECE-1, ET_A, ET_B, and AS mRNA in all samples examined (Fig. 1, A and B). The specificity of the amplification products obtained was confirmed by 1) hybridization with cDNA-specific probes, 2) size identity, and 3) lack of amplification of each cDNA when diethylpyrocarbonate-water was used instead of mRNA as the template for RT-PCR.

Immunocytochemistry

Cell monolayers showed a strong ET-1 immunoreactivity when incubated with either the polyclonal (not shown) or the monoclonal anti-ET-1 primary antibody (Fig. 2A), whereas no staining was seen when the primary antibody was omitted (Fig. 2B).

Autoradiographic studies

Specific binding of [^{125}I]ET-1 was demonstrated by comparison of total binding (Fig. 3A) and binding in the presence of excess cold ET-1 (Fig. 3B). The binding was partially displaced by both the ET_A antagonist BQ-123 (Fig. 3C) and the ET_B antagonists BQ-788 (Fig. 3D). Quantitative densitometric analysis of the autoradiograms revealed that the ET_B subtype accounted for the majority (mean ± SEM, 72.6 ± 9%) and the ET_A accounted for the minority (37.4 ± 5%) of the total [^{125}I]ET-1-specific binding.

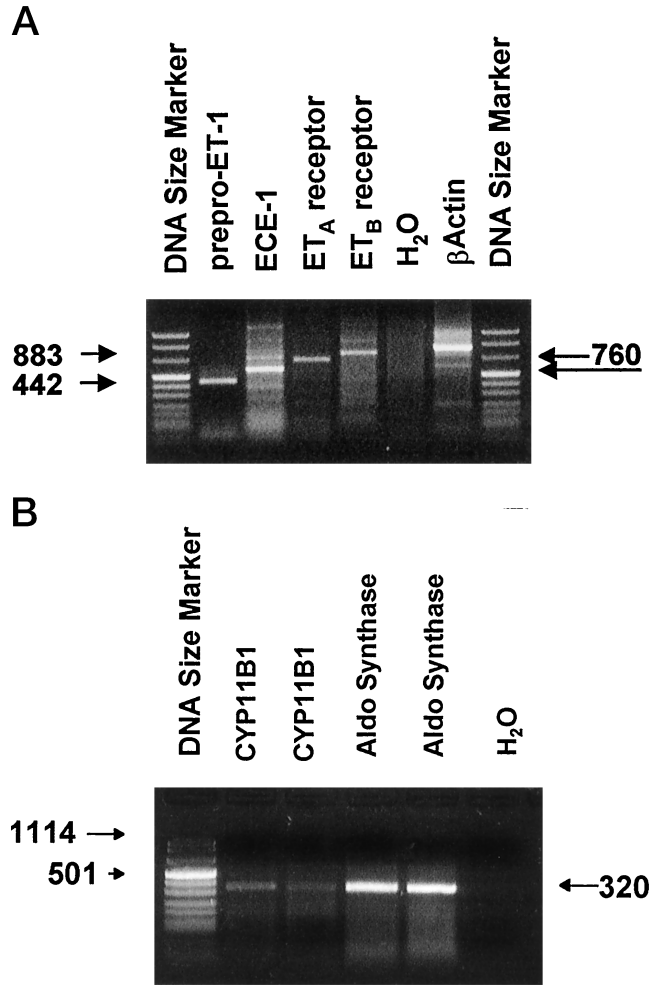

FIG. 1. A, Ethidium bromide-stained 1.5% agarose gel showing cDNA amplified with human prepro-ET-1-, hECE-1-, ET_A-, and ET_B-specific primers from NCI-H295. Lane 1 was loaded with 200 ng of a size marker (Boehringer Mannheim; marker VIII). The amplified fragments were of the expected sizes: 442 bp for prepro-ET-1, 567 bp for hECE-1, 669 bp for ET_A, and 760 bp for ET_B. No amplification of the PCR mixture with no cDNA template is also shown as a negative control. Amplification of a 838-bp fragment of β-actin is shown as a positive control. B, Ethidium bromide-stained 1.5% agarose gel showing cDNA amplified with human CYP11B1- and AS (CYP11B2)-specific primers from NCI-H295. The amplified fragments were of the expected sizes: 320 bp for both cDNAs. No amplification of the PCR mixture with no cDNA template is also shown as a negative control.

Effect of ET-1 on expression of the AS gene

The kinetics of amplification of the AS cDNA are reported in Fig. 4, where the amount of amplified DNA (log integrated optical density units) is plotted *vs.* the number of PCR cycles. The curve corresponding to the ET-1-treated cells cDNA was shifted to the left compared with that in the controls (Fig. 4), thereby indicating a higher initial abundance of AS mRNA (*i.e.* an enhanced expression of the AS gene) (23); however, the slopes of all curves were similar, suggesting a similar efficiency of amplification. The specific ET_B agonist IRL-1620 displayed a similar shift to the left compared with the control curve. The concomitant

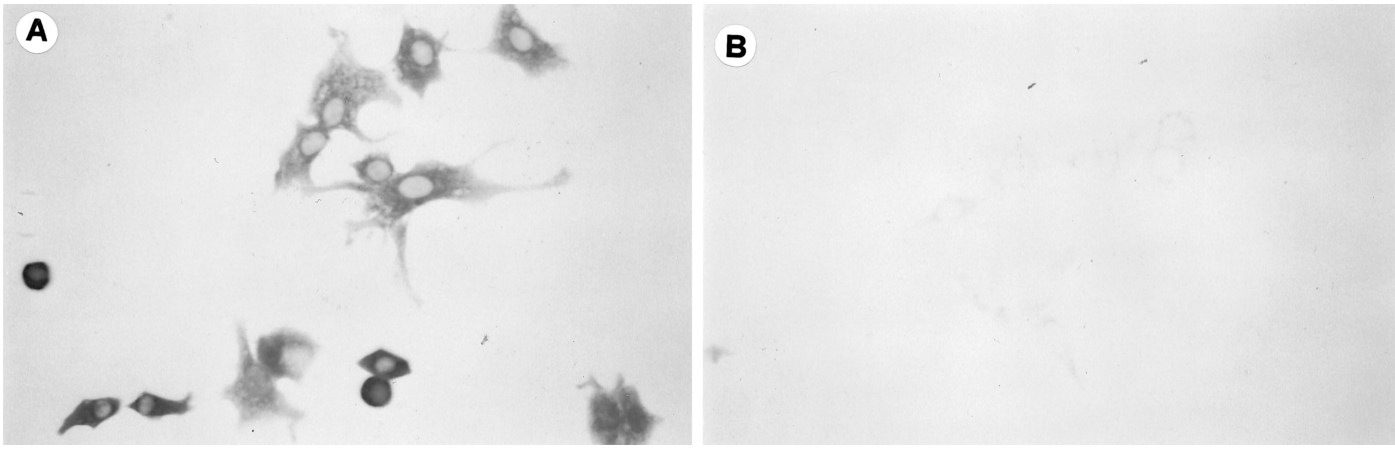

FIG. 2. The results of immunohistochemistry confirm the presence of mature ET-1 in NCI-H295 cells. Cell monolayers showed a strong ET-1 immunoreactivity when incubated with the monoclonal mouse ET-1 primary antibody (A), whereas no staining was seen when the primary antibody was omitted (B).

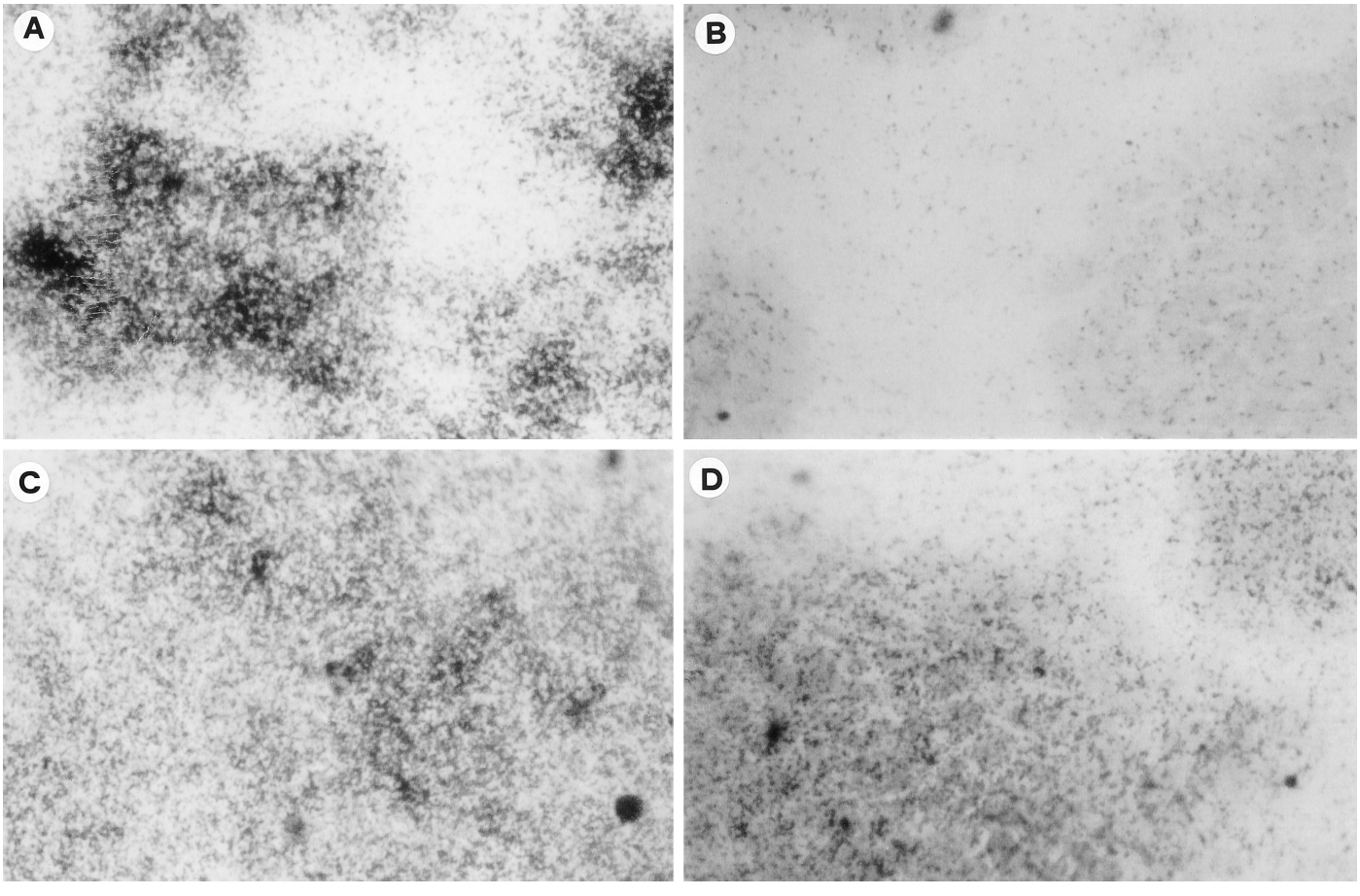

FIG. 3. Autoradiographs of monolayer of human adrenocortical carcinoma cells incubated with [^{125}I]ET-1 (100 pM). [^{125}I]ET-1 binding is intense to the cells (A) and is completely displaced by the addition of 1 mM cold ET-1 (B). Both the ET_A antagonist BQ-123 (500 mM) (C) and the ET_B agonist sarototoxin 6C (500 mM) (D) attenuate [^{125}I]ET-1 binding to the cells, thereby confirming the presence of both receptor subtypes. Magnification is ×160 for all sections.

exposure to ET-1 and either BQ-123 or BQ-788 did not abolish the stimulatory effect of ET-1. The N_{50}, *i.e.* the number of cycles at which half-maximal amplification was attained, was significantly lower for ET-1 (25.00 ± 0.24 SEM; $P = 0.006$) and IRL-1620 (26.18 ± 0.59; $P < 0.05$) than for the controls (28.90 ± 0.20). Similarly, the N_{50} of cells treated with both ET-1 plus BQ-788 (25.61 ± 0.84) and ET-1 plus BQ-123 (26.04 ± 0.70) was lower than the control value, although not significantly so, due to the larger dispersion of the data.

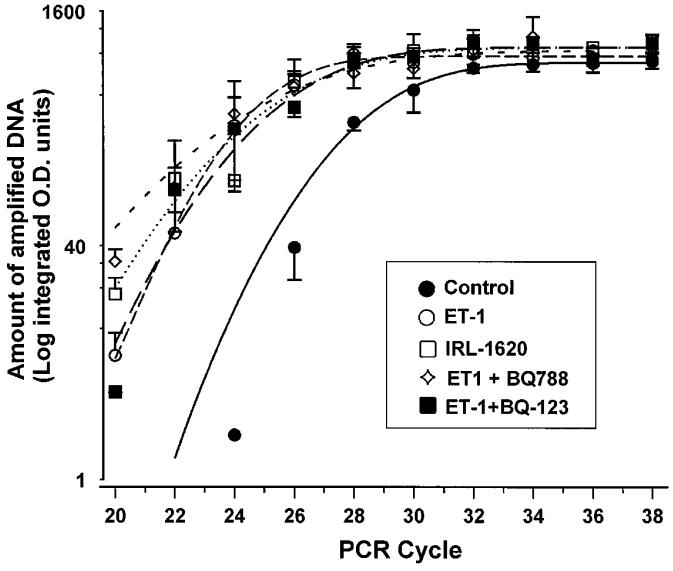

FIG. 4. Kinetics of PCR amplification of AS cDNA from NCI-H295 showing the effect of ET-1, IRL-1620, ET-1 plus BQ-123, and ET-1 plus BQ-788. The N_{50}, *i.e.* the number of cycles at which half-maximal amplification was attained, was significantly lower for ET-1 (P = 0.006) and IRL-1620 than for controls, thereby demonstrating a greater abundance of specific mRNA in cells treated with these agonists. Neither BQ-123 nor BQ-788 was able to abolish the ET-1-induced enhancement.

Effect of ET-1 on $[Ca^{2+}]_i$

The exposure of NCI-H295 cells to ET-1 induced a 95% increase in $[Ca^{2+}]_i$ (from 64.3 ± 6 to 127.3 ± 19 SEM; n = 4; Fig. 5A). This effect rapidly disappeared with repeated exposure to ET-1 and was abolished by 10-min pretreatment with the BQ-788 (Fig. 5B), whereas it was not affected by pretreatment with BQ-123 (Fig. 5C). Either BQ alone did not modify resting $[Ca^{2+}]_i$. The exposure of the cells to IRL 1620 induced a 91% increase in $[Ca^{2+}]_i$ (from 65 ± 7 to 124 ± 22; n = 3; Fig. 5D). This effect was prevented by 10-min pretreatment with BQ-788 (data not shown).

Discussion

Identification of the functional role of ETs and their receptor subtypes in the regulation of aldosterone biosynthesis and secretion in humans has been prevented to date by the lack of suitable models of investigation. From this standpoint, the availability of a cell line capable of synthesizing aldosterone in a regulated fashion and expressing both ET receptor subtypes would be very desirable.

Our coupled cell biology, immunocytochemical, and autoradiographic study shows that NCI-H295 cells not only express the mRNAs of prepro-ET-1 and hECE-1 and can synthesize immunoreactive ET-1, a finding in partial agreement with the results of Li *et al.* (28) in adrenocortical carcinomas, but also that they can express and translate into functional proteins both ET_A and ET_B receptor subtypes. Thus, these results strongly suggest that ET-1 may act as an autocrine-paracrine factor in the regulation of NCI-H295 cell function.

This contention is supported by the demonstration that

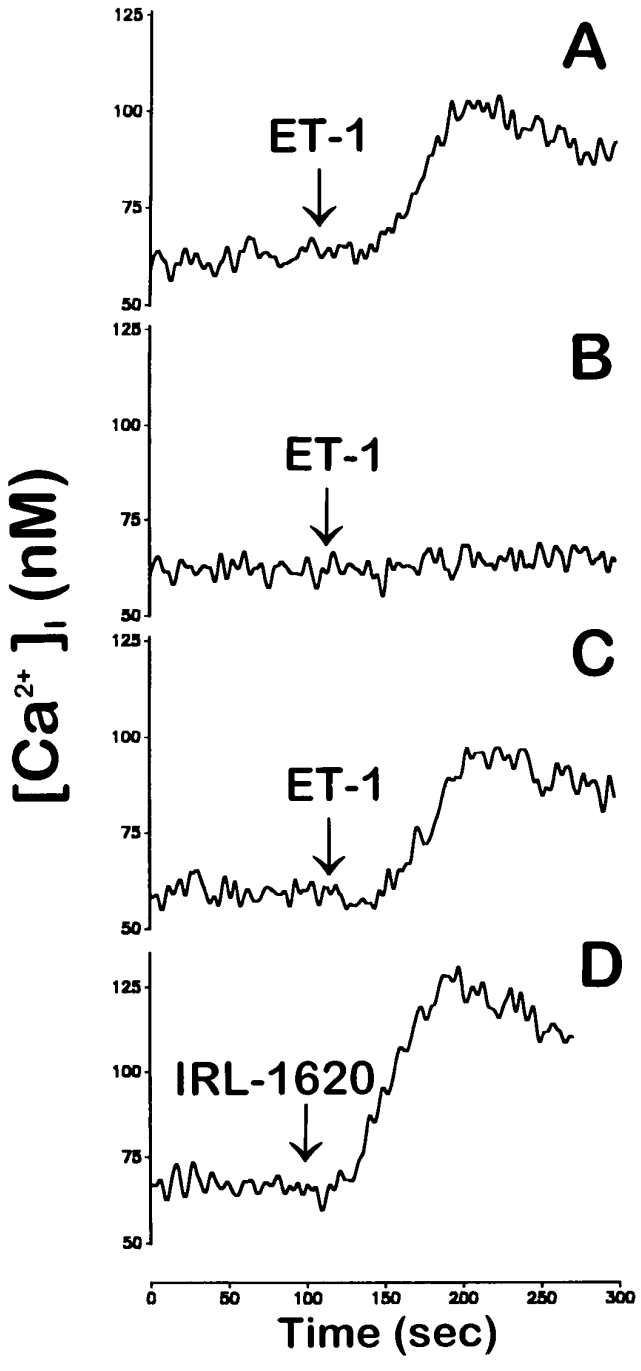

FIG. 5. Effect of ET-1 (10 nM) (A) on $[Ca^{2+}]_i$ in NCI-H295 cells. B, Ten-minute pretreatment with BQ-788 (0.8 mM) abolished the increase in $[Ca^{2+}]_i$, whereas 10-min pretreatment with BQ-123 (1 mM) was ineffective (C). The ET_B agonist IRL 1620 (100 nM) mimicked the effect of ET-1 (D). No change in resting $[Ca^{2+}]_i$ was induced by either BQ-788 or BQ-123.

ET-1 is able to enhance the expression of the AS gene through a mechanism that is likely to mainly involve the ET_B receptor subtype. In fact, our findings indicate that ET-1 exposure induces a higher initial abundance of AS mRNA in the NCI-H295 cells, and the ET_B agonist IRL-1620 is equipotent to ET-1 as far as this effect is concerned. It must be pointed out, however, that exposure to either the specific ET_A antagonist

BQ-123 or the specific ET_B antagonist BQ-788 alone could not abolish the stimulatory effect of ET-1, thereby suggesting that both receptor subtypes are somehow involved in the mediation of this effect of ET-1.

As concerns the intracellular event brought about by ET receptor activation, we found that activation of the ET_B receptor subtype by either ET-1 or IRL 1620 leads to a clear-cut increase in $[Ca^{2+}]_i$, which vanished with repeated ET-1 stimulation, probably due to homologous desensitization (27). This effect was abolished by pretreating the cells with the specific ET_B antagonist BQ-788 alone or in combination with BQ-123, but not by treatment with the specific ET_A antagonist BQ-123 alone, thereby confirming that the ET-1-induced increase in $[Ca^{2+}]_i$ is an ET_B receptor-mediated effect. Calcium mobilization is deemed to play an important role in the control of steroidogenesis in the adrenal cortex (29); accordingly, the bulk of the evidence indicates that activation of the ET_B receptor subtype mediates the aldosterone secretagogue effect of ET-1 (for review, see Ref. 8).

Hence, the question arises of what are the cellular events associated with ET_A receptor subtype activation and ensuing stimulation of AS gene expression. The fact that no evident increase in $[Ca^{2+}]_i$ and aldosterone secretion was elicited by ET-1 activation of the ET_A subtype is intriguing and obviously worthy of further investigation.

In conclusion, our results provide evidence for the concomitant expression AS and the prepro-ET-1, hECE-1, ET_A, and ET_B receptor genes in NCI-H295 adrenocortical carcinoma-derived cells. Furthermore, they demonstrate that NCI-H295 cells synthesize mature ET-1 and express both the ET_A and ET_B receptor subtypes at the protein level. Both ET receptors are able to bind $[^{125}I]$ET-1 and can mediate the secretagogue effect of ET-1 on aldosterone by acting in an autocrine-paracrine fashion at the transcriptional level of AS; however, only ET_B activation was found to be associated with a sizable increase in $[Ca^{2+}]_i$. Thus, these findings indicate that NCI-H295 cells may be a useful model for investigating the role of ET-1 and its receptor subtypes in the regulation of adrenocortical function in humans as well as the potential autocrine-paracrine role of ETs in the pathogenesis of adrenal tumors.

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References

1. **Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T** 1988 A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411–415
2. **Kennedy RL, Haynes WG, Webb DJ** 1993 Endothelins as regulators of growth and function in endocrine tissues. Clin Endocrinol (Oxf) 39:259–265
3. **Rubanyi GM, Polokoff MA** 1994 Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev 46:325–415
4. **Battistini B, D'Orleans Juste P, Sirois P** 1993 Endothelins: circulating plasma levels and presence in other biologic fluids. Lab Invest 68:600–628
5. **Rossi GP, Albertin G, Belloni A, Zanin L, Biasolo MA, Prayer Galetti T, Bader M, Nussdorfer GG, Palù G, Pessina AC** 1994 Gene expression, localization, and characterization of endothelin A and B receptors in the human adrenal cortex. J Clin Invest 94:1226–1234
6. **Belloni AS, Rossi GP, Andreis PG, Neri G, Albertin G, Pessina AC, Nussdorfer GG** 1996 The ET_B receptor subtype mediates the steroid secretagogue response in the rat adrenal cortex. Hypertension 27:1153–1159
7. **Rossi GP, Belloni AS, Albertin G, Zanin L, Biasolo MA, Nussdorfer GG, Palù G, Pessina AC** 1995 Endothelin-1 and its receptors A and B in human aldosterone-producing adenomas. Hypertension 25:842–847
8. **Nussdorfer GG, Rossi GP, Belloni AS** 1997 The role of endothelins in the paracrine control of the secretion and growth of the adrenal cortex. Int Rev Cytol 171:267–308
9. **Gazdar AF, Oie HK, Shackleton CH, Chen TR, Triche TJ, Myers CE, Chrousos GP, Brennan MF, Stein CA, La Rocca RV** 1990 Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res 50:5488–5496
10. **Bird IM, Hanley NA, Word RA, Mathis JM, McCarthy JL, Mason JI, Rainey WE** 1993 Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion. Endocrinology 133:1555–1561
11. **Bird IM, Mason JI, Rainey WE** 1994 Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells. Endocrinology 134:2468–2474
12. **Rainey WE, Bird IM, Mason JI** 1994 The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. Mol Cell Endocrinol 100:45–50
13. **Bird IM, Imaishi K, Pasquarette MM, Rainey WE, Mason JI** 1996 Regulation of 3β-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. J Endocrinol 150:S165–S173
14. **Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, Fukami T, Ishikawa K, Nishikibe M, Yano M** 1992 Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. Life Sci 50:247–255
15. **Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T, Fukuroda T, Fukami T, Ozaki S, Nagase T, Nishikibe M, Yano M** 1994 Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc Natl Acad Sci USA 91:4892–4896
16. **Takasaki C, Yanagisawa M, Kimura S, Goto K, Masaki T** 1988 Similarity of endothelin to snake venom toxin. Nature 335:303–304
17. **Takai M, Umemura I, Yamasaki K, Watakabe T, Fujitani Y, Oda K, Urade Y, Inui T, Yamamura T, Okada T** 1992 A potent and specific agonist, Suc-[Glu^9,$Ala^{11,15}$]-endothelin-1(8–21), IRL 1620, for the ETB receptor. Biochem Biophys Res Commun 184:953–959
18. **Rossi GP, Albertin G, Franchin E, Sacchetto A, Cesari M, Palù G, Pessina AC** 1995 Expression of the endothelin-converting enzyme gene in human tissues. Biochem Biophys Res Commun 211:249–253
19. **Dirnhofer S, Berger C, Untergasser G, Geley S, Berger P** 1995 Human β-actin retropseudogenes interfere with RT-PCR. Trends Genet 11:380–381
20. **Pagotto U, Arzberger U, Hopfner U, Sauer U, Renner U, Newton CJ, Lange M, Uhl E** 1995 Expression and localization of endothelin receptors in human meningiomas: evidence for a role in tumoral growth. J Clin Invest 79:1864–1870
21. **Kuhar MJ** 1985 Receptor localization with microscope. In: Yamamura HI, Enna SJ, Kuhar MJ (eds) Neurotransmitter Receptor Binding. Raven Press, New York, pp 153–176
22. **Curnow KM, Tusie-Luna MT, Pascoe L, Natarajan R, Gu JL, Nadler JL, White PC** 1991 The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. Proc Natl Acad Sci USA 5:1513–1522
23. **Trapnell BC** 1993 Quantitative evaluation of gene expression in freshly isolated human respiratory epithelial cells. Am J Physiol 264:L199–L212
24. **Bird IM, Word RA, Clyne C, Mason JI, Rainey WE** 1995 Potassium negatively regulates angiotensin II receptor expression in human adrenocortical H295R cells. Hypertension 25:1129–1134
25. **Bova S, Trevisi L, Debetto P, Cima L, Furneri M, Luciani S, Padrini R, Cargnelli G** 1996 Vasorelaxant properties of norbormide, a selective vasoconstrictor agent for the rat microvasculature. Br J Pharmacol 117:1041–1046
26. **Grynkiewicz GM, Poenie M, Tsien RY** 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
27. **Cozza EN, Gomez Sanchez CE** 1990 Effects of endothelin-1 on its receptor concentration and thymidine incorporation in calf adrenal zona glomerulosa cells: a comparative study with phorbol esters. Endocrinology 127:549–554
28. **Li Q, Grimelius L, Grondal S, Hoog A, Johansson H** 1994 Immunohistochemical localization of endothelin-1 in non-neoplastic and neoplastic adrenal gland tissue. Virchows Arch 425:259–264
29. **Ganguly A, Davis JS** 1994 Role of calcium and other mediators in aldosterone secretion from the adrenal glomerulosa cells. Pharmacol Rev 46:417–447