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Characterization of Endothelin-Converting Enzyme from Endothelial Cells and Rat Brain: Detection of the Formation of Biologically Active Endothelin-1 by Rapid Bioassay

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SUMMARY

Using the endothelin-1 (ET-1)-stimulated elevation in cGMP in LLC-PK_1 cells as a biological detector system for the conversion of big ET-1 (bET-1) to ET-1, we detected bET-1-converting activities in subcellular fractions from bovine aortic cultured endothelial cells (BAE) and rat brain. Within the particulate fraction of BAE, we detected two activities, at pH 3.4 and pH 5.4–7.4. The latter but not the former activity was inhibited in a concentration-dependent manner by phosphoramidon (approximate IC_{50}, 1 μM) and converted bET-1 to ET-1 at a rate of 0.6 nmol/hr/mg of protein. It could be solubilized from the particulate fraction by detergent treatment. Phosphoramidon-inhibitable converting activity was also detected in the cytosolic fraction of BAE. Within the rat brain, phosphoramidon-inhibitable conversion of bET-1 to ET-1 was detected principally in the cytoskeletal fraction, i.e., that fraction from the membrane that was not solubilized by detergent treatment. These results show the presence of at least two different endothelin-converting enzyme activities in endothelial cells and a third within the rat brain. They also demonstrate the use of LLC-PK_1 cells as a rapid assay that permits the sensitive detection and measurement of the formation of biologically active ET-1 from its precursor bET-1.

The biosynthetic pathway involved in the formation of the 21-amino acid peptide ET-1 (1) appears to involve the cleavage of the 38- or 39-amino acid precursor bET-1 by a specific ECE. Current evidence suggests that there are at least two ECE activities present in endothelial cells. One of these is an aspartic protease or cathepsin-like enzyme that is active at pH 3.5 and inhibitable by pepstatin A (2, 3). The other is a metalloendopeptidase that is active at neutral pH and inhibitable by phosphoramidon (4, 5). This second enzyme may be of more importance physiologically for the conversion of bET-1 to ET-1, because phosphoramidon blocks the pressor, airway contractile, and prostanoid-releasing effects of bET-1 *in vivo* (6–8) and inhibits the release of ET-1 from cultured endothelial cells (9, 10). Experiments to examine the conversion of bET-1 to ET-1 rely either on separation of these two peptides by high performance liquid chromatography or on selective radioimmunoassay. Both of these methods have drawbacks, in that high performance liquid chromatography requires relatively large amounts of substrate and enzyme, and radioimmunoassays are usually complicated by cross-reactivity between the substrate (bET-1) and the product (ET-1), with the anti-ET-1 antibodies. In addition, neither shows the generation of the biologically active form of ET-1 from bET-1. Here, by using a simple and selective bioassay, we are able to demonstrate the rapid conversion of bET-1 to ET-1, within 20 min, by crude particulate or cytosolic fractions of BAE. This conversion is pH, time, and protein dependent. We were also interested in examining the conversion of bET-1 to ET-1 within the brain, because evidence has suggested the presence in this tissue of the endothelin biosynthetic pathway (11) and the conversion of exogenous bET-1 to ET-1 (12). Here we show that there is a phosphoramidon-sensitive ECE within the cytoskeletal fraction isolated from rat brains. Thus, by bioassay, we can detect ECE activity within both BAE and rat brain. This assay is also highly selective, because it relies upon the 10,000-fold difference in potency of bET-1 and ET-1 to stimulate the accumulation of cGMP within LLC-PK_1 porcine kidney epithelial cells (PK_1 cells) (13).

Materials and Methods

Cell culture. BAE (National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository, Camden NJ) were cultured

ABBREVIATIONS: ET-1, endothelin-1; bET-1, big endothelin-1; ELISA, enzyme-linked immunosorbent assay; BAE, bovine aortic cultured endothelial cells; ECE, endothelin-converting enzyme; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate.

in roller bottles containing Eagle's minimum essential medium supplemented with 20% fetal bovine serum and minimum essential medium nonessential amino acids (0.1 mM each), at 37°. PK_1 cells (American Type Culture Collection CL 101) were grown to confluency in six-well plates (well diameter, 35 mm), in medium 199 with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, at 37° under an atmosphere of 95% air/5% CO_2 (14).

Preparation of rat brains. Rats (male Sprague-Dawley, 250–400 g) were rendered unconscious with CO_2 and decapitated. The brains were rapidly removed and frozen in liquid N_2.

Preparation of subcellular fractions. BAE were gently scraped from the roller bottles, washed in phosphate-buffered saline, and suspended in buffer A (50 mM HEPES, 100 mM NaCl, pH 7.4). Rat brains were thawed in 5 volumes of buffer A. The tissues were then homogenized on ice, by using a glass tissue grinder with a Teflon pestle, and the homogenates were centrifuged at 100,000 × *g* for 1 hr. Subsequent fractions were prepared as described previously (15). Briefly, the pellet fraction was washed once with buffer A and resuspended in buffer A by homogenization. In some experiments, the particulate fraction was washed with 1 M KCl (in buffer A) for 5 min at 4°. After centrifugation (100,000 × *g* for 30 min), the pellet was resuspended in buffer A. The particulate fraction was then treated with the detergent CHAPS (20 mM) for 20 min at 4°. After centrifugation (100,000 × *g* for 30 min), the supernatant was collected (CHAPS-solubilized pellet fraction) and the pellet was resuspended in buffer A (cytoskeletal fraction). All fractions were assayed for ECE activity. Protein was determined using the Bradford reagent (Bio-Rad), with bovine serum albumin as the standard. Aliquots of the subcellular fractions were stored at −70°.

Assay of ECE activity. BAE or rat brain protein (2.5–80 μg of protein) was incubated (37°, 0–20 min) at pH 7.4 (unless otherwise stated), in the absence or presence of phosphoramidon (0.1–100 μM) and/or bET-1 (6–600 pmol), in a total volume of 25 μl in buffer A. The incubates were then rapidly frozen in liquid N_2 and stored (no more than 2 hr) at −20°. After rapid thawing, the incubate was added to PK_1 cells for 4 min, as described below. The concentration of ET-1 present in the sample was then calculated from the levels of cGMP produced in the PK_1 cells, compared with the levels produced by known concentrations of ET-1.

Endothelin ELISA. In some experiments, the amounts of ET-1 formed in incubates of bET-1 and the test protein fractions were measured by specific ELISA, using antibody directed against the carboxyl-terminal fragment of ET-1 [$ET\text{-}1_{(17\text{-}21)}$], with approximately 0.01% cross-reactivity with bET-1. Binding was detected by peroxidase-labeled goat anti-rabbit IgG antibody, using *o*-phenylenediamine-2HCl as a substrate. The absorbance of each well was measured at 490 nm, using a microplate reader (Bio-Tek model EL311).

Measurement of cGMP levels in PK_1 cells. Before experimentation, the culture medium was removed and the cells were washed twice with 2 ml of Locke's solution (pH 7.4), of the following composition (in mM): NaCl, 154.0; KCl, 5.6; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 3.6; glucose, 5.6; HEPES, 10.0; and 3-isobutyl-1-methylxanthine, 0.3. Cells were equilibrated for 20 min in 1 ml of the same buffer. Experiments were conducted at 37° with gentle shaking. After the cells were exposed to either ET-1 standards or ECE/bET-1 mixtures for 4 min, the incubation buffer was removed and reactions were stopped by addition of 1 ml of ice-cold sodium acetate buffer (50 mM, pH 4.0). Samples were then quickly frozen with liquid N_2 (11). After thawing of the frozen samples at room temperature, the content of cGMP in the cells was determined by radioimmunoassay (16).

Chemicals. 3-Isobutyl-1-methylxanthine and phosphoramidon [*N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp] were purchased from Sigma Chemical Co. (St. Louis, MO). bET-1 (big endothelin, human) and ET-1 (human, porcine) were obtained from Peninsula Laboratories, Inc. (Belmont, CA). All other chemicals and reagents used were of the highest quality available.

Statistics. Results are shown as mean ± standard error for *n* experiments. Student's unpaired *t* test was used to determine the significance of differences, and a *p* value of <0.05 was taken as significant.

Results and Discussion

Calibration of ET-1 response on PK_1 cells. ET-1 (10^{-11} to 10^{-7} M, 10 fmol to 100 pmol/well, for 4 min) caused a concentration-dependent increase in the cGMP levels in the PK_1 cells ($n > 20$). The linear portion of the cGMP dose-response curve to ET-1 was then used to calculate the amount of ET-1 in each test sample assayed. bET-1, up to a concentration of 10^{-7} M (100 pmol/well), did not cause significant stimulation of cGMP levels in the PK_1 cells ($n = 5$).

Subcellular location of ECE activity within BAE. After fractionation of the BAE, phosphoramidon-inhibitable ECE activity was detected in both the cytosolic (17) and particulate (5) fractions at neutral pH (Fig. 1). The activity was greatest in proteins solubulized from the membrane fraction by treatment with CHAPS, which may reflect detergent activation of the enzyme or expression of latent enzyme activity after solubilization. This observation is consistent with the report that the ECE activity from the particulate fraction of BAE could be solubilized by treatment with the detergent Triton X-100 (5). Although we also detected a cytosolic activity that was similar to that of the particulate fraction in terms of specific activity, the particulate fraction contained significantly more activity when calculated as total activity (28.8 nmol/hr versus 15.0 nmol/hr for conversion of bET-1 to ET-1). We, therefore, used the particulate fraction as the source for further enzyme characterization. In subsequent experiments to confirm the presence of ECE by ELISA, we found that we could detect phosphoramidon-inhibitable activity within the particulate fraction (data not shown). This activity was up to 2 times greater than that recorded by parallel bioassay and was only inhibited by 56 ± 8% ($n = 6$) in the presence of phosphoramidon. This suggests that the particulate fraction contains other activities that are capable of producing bET-1 fragments that cross-react with the antibody but do not produce biological effects.

Protein and substrate dependence of ECE activity from BAE. Incubation of bET-1 (6–60 pmol) and crude BAE

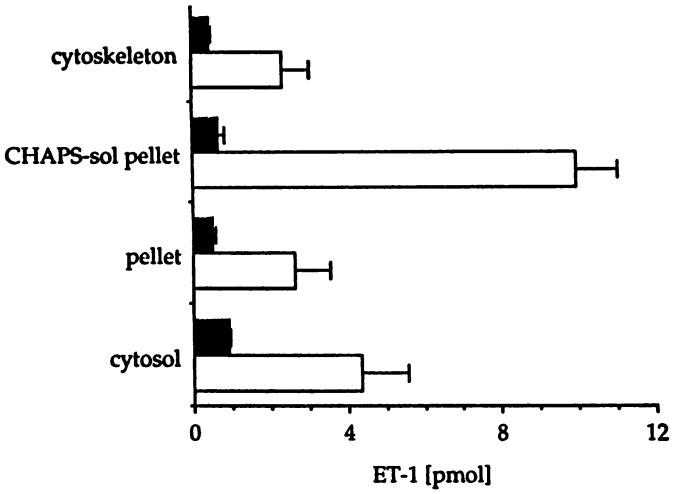

Fig. 1. Localization of ECE activity in BAE fractions. BAE fractions (8 μg of protein) were incubated (37°) with bET-1 (60 pmol) for 20 min either in the absence (□) or in the presence (■) of phosphoramidon (100 μM). Data represent the mean ± standard error ($n = 3$) from a representative experiment.

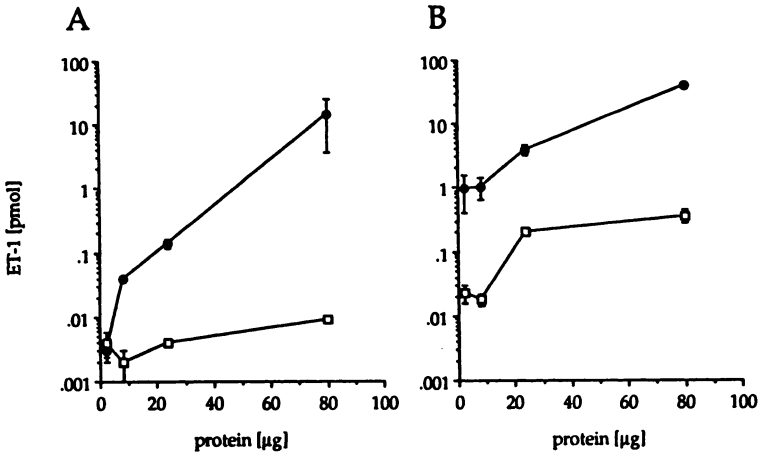

Fig. 2. Production of ET-1 from bET-1 by ECE from BAE is protein and substrate dependent. The particulate fraction of BAE was incubated at a concentration of 2.4–80 μg of protein/25 μl with bET-1 at a concentration of either 6 pmol/25 μl (A) or 60 pmol/25 μl (B). All incubations were for 20 min at pH 7.4, in the absence (●) or presence (□) of phosphoramidon (100 μM). Each *point with a vertical bar* represents the mean ± standard error (n = 3).

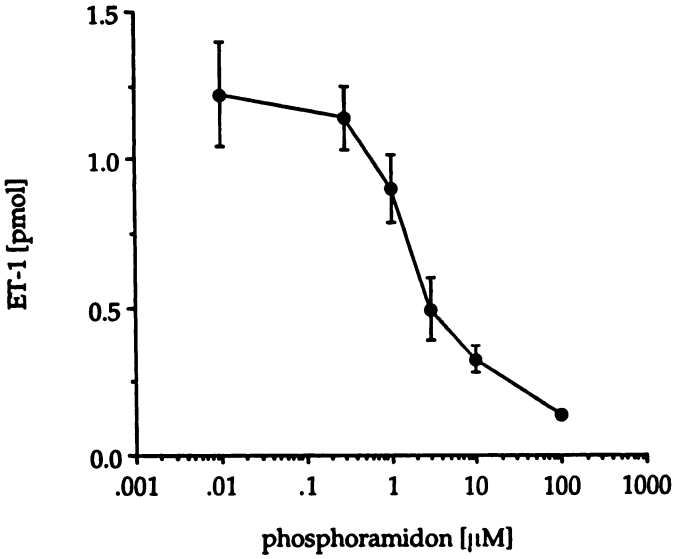

Fig. 3. Inhibition of BAE ECE activity by phosphoramidon. BAE particulate fraction (8 μg of protein) was incubated (37°) with bET-1 (60 pmol) for 20 min in the presence of phosphoramidon (0.01–100 μM). Each *point with a vertical bar* represents the mean ± standard error (n = 3).

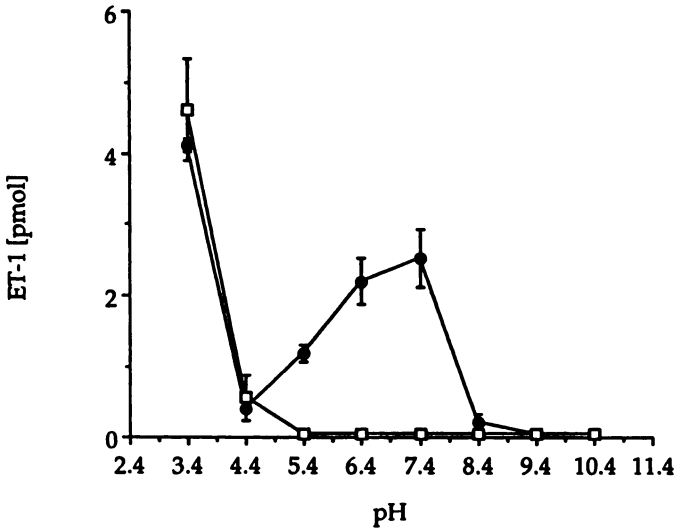

Fig. 4. pH dependence of the conversion of bET-1 to ET-1. BAE particulate fractions (8 μg of protein) were incubated (37°) with bET-1 (60 pmol) for 20 min at pH 3.4–10.4. Two activities converted bET-1 to ET-1 (●). One was active at pH 3.4 and the other at pH 5.4–7.4. The latter but not the former was completely inhibited in the presence of phosphoramidon (100 μM) (□). Each *point with a vertical bar* represents the mean ± standard error (n = 3).

pellet (2.5–80 μg) in a volume of 25 μl at pH 7.4 led to protein- and substrate-dependent formation of ET-1 (Fig. 2). It was not possible to measure the conversion of bET-1 (600 pmol) by bioassay, because this amount of bET-1 directly stimulates the PK$_1$ cells (13). However, when conversion was assayed by ELISA, we did not find any increase in ET-1 formation (t = 5–60 min) in the presence of 600 pmol compared with 60 pmol of bET-1/8 μg of protein (0.08 versus 0.07 pmol/min). Phosphoramidon caused a concentration-dependent inhibition of this ECE activity, with an IC$_{50}$ of approximately 1 μM (Fig. 3). We also found that phosphoramidon inhibited the ECE activity immediately any time it was added to the incubate after the addition of bET-1; thus, the inhibition was rapid in onset, without a time lag. Preincubation of cellular extracts with phosphoramidon before the addition of bET-1 did not increase the inhibition (data not shown). These observations are in accordance with the suggested mode of action of phosphoramidon as a competitive inhibitor of ECE (17).

At higher concentrations of cell extract, ET-1 formation independent of additional substrate was detected (data not shown). This was inhibitable by phosphoramidon in a time-dependent manner and was presumably due to the presence of small amounts of bET-1 within the BAE at the time of harvesting.

pH dependence of ECE activity within BAE. Over a pH range of 3.4–10.4, we detected two ECE activities (Fig. 4). One activity was present only below pH 4.4 and was not inhibited by phosphoramidon. This is most probably very similar to the low pH, pepstatin A-inhibitable activity described from endothelial cells (2, 3). The second activity was demonstrable over a pH range of 5.4–7.4, with a peak of activity at pH 7.4, and was completely inhibited by phosphoramidon at a concentration of 100 μM. Because the release of mature ET-1 from BAE is inhibited by phosphoramidon (9, 10), it is very likely that this ECE represents the physiologically relevant activity for the processing of bET-1 to ET-1. The activity that we studied was maximal at pH 7.4, but we could still detect ECE activity at pH 5.4, 1 pH unit lower than that reported previously for the phosphoramidon-inhibitable ECE (5, 17).

Time dependence of ECE activity within BAE. The

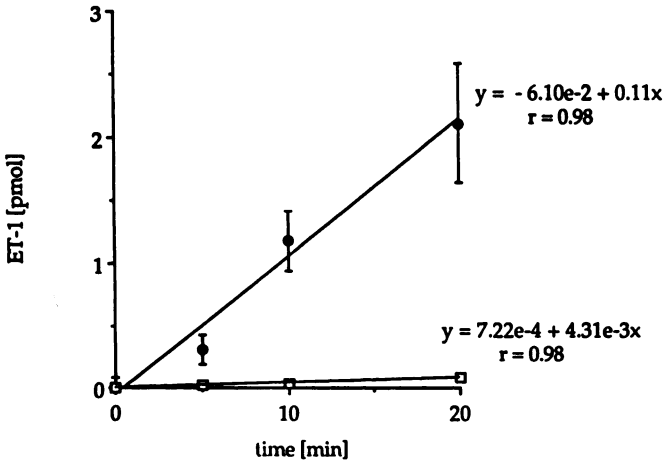

Fig. 5. Time dependence of bET-1 conversion to ET-1. BAE particulate fraction (8 μg of protein) was incubated (37°) with bET-1 (60 pmol) for 0–20 min, at pH 7.4. The production of ET-1 from bET-1 was linear over this time period (●). In the presence of phosphoramidon (100 μM) (□), the conversion of bET-1 to ET-1 was reduced by >90%. Each *point with a vertical bar* represents the mean ± standard error (n = 3).

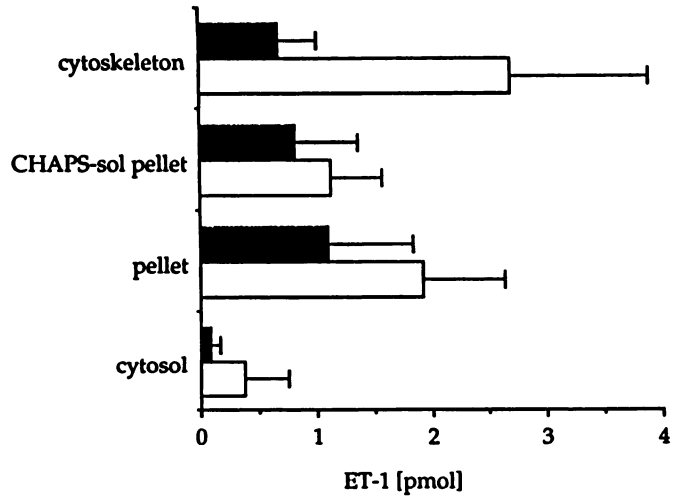

Fig. 6. Localization of ECE activity in rat brain fractions. Figure shows the calculated specific activity of ECE within fractions prepared from rat brain; 8 μg of protein were incubated (37°) with bET-1 (60 pmol) for 20 min either in the absence (□) or in the presence (■) of phosphoramidon (100 μM). *Bars* represent the mean ± standard error of data from three separate experiments, in each of which the samples were assayed in triplicate.

formation of ET-1 from bET-1, as assayed over 0–20 min, was linear in our system (Fig. 5). When the amount of bET-1 supplied to the ECE was saturating (60 pmol/8 μg of protein), we found, in three separate determinations, the rate of formation of ET-1 in the crude pellet fraction to be 0.61 nmol/hr/mg of protein. This rate appears relatively low for the formation of a physiologically relevant mediator, but this may be explained by the impurity of the ECE sample and by the fact that the very long duration of response to ET-1 (1) does not necessitate a rapid turnover of substrate. In the presence of phosphoramidon (100 μM), the rate of conversion was decreased to 0.014 nmol/hr/mg of protein, i.e., a reduction in activity of >97%.

Subcellular location of ECE activity within rat brain. After fractionation of the rat brain, ECE activity that was significantly inhibited by phosphoramidon was detected predominantly in the cytoskeletal fraction at neutral pH (Fig. 6). This contrasts with the BAE and suggests that the activity isolated from brain may not be derived from endothelial cells present within the tissue. This would be in accordance with the suggestion that the endothelin synthetic pathway may be situated predominantly within astroglial cells (11). Specific ELISA for ET-1 confirmed that 25 μg of cytoskeletal protein converted 350 pmol of bET-1 to 1.2 ± 0.01 pmol of ET-1 in 20 min. Data derived by bioassay demonstrated that 8 μg of cytoskeletal protein converted 60 pmol of bET-1 to 1.4 ± 0.5 pmol of ET-1 in the same time period. In both experimental systems, this conversion was inhibited significantly in the presence of phosphoramidon (100 μM), by >65%. Thus, the cytoskeletal fraction from rat brain contains phosphramidon-inhibitable ECE.

In conclusion, using the formation of cGMP within PK_1 cells as an assay for ECE activity we are able to detect the formation of biologically active product from bET-1 by ECE. This enzyme activity is functional at neutral pH, inhibitable by phosphoramidon, and situated in the particulate fraction of both BAE and rat brain. In the case of BAE, this most probably represents the physiologically relevant ECE. However, it is not clear whether the activity within the rat brain cytoskeleton fraction is also physiologically relevant. The fact that it is contained within a different subcellular fraction than the ECE from BAE suggests that it may be derived from a brain cell type other than endothelial.

In addition to our findings on BAE and rat brain ECE, we have demonstrated that the use of PK_1 cells as a bioassay permits us to assay the conversion produced by relatively low concentrations of protein, within a time period of 20 min, and there is no significant cross-reaction with the substrate bET-1. Thus, this assay could be very useful for experiments to purify and characterize the ECE.

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