Biochemical and Biophysical Research Communications **261,** 149–155 (1999)
Article ID bbrc.1999.0924, available online at http://www.idealibrary.com on IDEAL®

Endothelin Converting Enzyme Activity in Primary Rat Astrocytes Is Modulated by Endothelin B Receptors

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Received May 24, 1999

Astrocytes express endothelin-1 (ET-1), ET-3, and their receptors, ET_A and ET_B. We report here that activated astrocytes *in vivo* also express endothelin converting enzyme-1 (ECE-1). Higher basal ET-1 concentrations in astrocyte media from ET_B-deficient (sl/sl) versus wild-type (+/+) rats suggested that altered ECE activity may be related to the absence of ET_B receptors. Quantification of ECE activity in membranes from sl/sl astrocytes yielded a 50% higher conversion compared to +/+ astrocytes, with indistinguishable ECE-1 mRNA and protein levels. Kinetic analysis of ECE activity revealed similar V_{max} values in sl/sl and +/+ astrocytes. Enzyme activity was competitively inhibited by phosphoramidon with K_i values of 0.6 and 0.3 μM, respectively. The K_m value of ECE was 0.5 μM in +/+ and 0.2 μM in sl/sl astrocytes. Two-dimensional focussing of astrocytic ECE-1 uncovered heterogeneity of charge and molecular weight. ECE-1 from sl/sl revealed a glycosylation pattern different from +/+ astrocytes. In conclusion, the ET_B receptor may, via ECE-1 glycosylation, exert a negative feedback on ECE activity in the astrocytic endothelin system.

Endothelins (ETs), namely ET-1, ET-2, and ET-3, are a family of potent vasoactive and growth-regulatory 21-amino acid peptides that act via G-protein coupled receptors, ET_A and ET_B, for review see (1–4). ET-1, ET-2, and ET-3 are encoded by three separate genes and differ from each other by two and six amino acids, respectively, and also by their biological properties (5). All three peptides are derived from larger precursor molecules, prepro-ET-1, -2, and -3, that are first cleaved to generate bigET-1, -2, and -3. These inactive intermediates have to be hydrolyzed by an endothelin converting enzyme (ECE) to give the final active products, mature ET-1, -2, and -3, for review see (6). This conversion provides an important regulatory step in endothelin biology.

ECE-1 (EC 3.4.24.71) has been cloned, functionally expressed, and is now established to be a type II membrane-bound, phosphoramidon-sensitive, highly glycosylated, neutral zinc metalloprotease (7–9). It belongs to the gluzincin family of metalloproteases and forms together with NEP 24.11 (EC 3.4.24.11), Kell blood group protein (EC 3.4.24.-), and Peptidase O (EC 3.4.24.-) the subfamily M13 (10). N-Glycosylation has been shown to be functionally important for ECE-1 and NEP 24.11 (11, 12). In the rat, ECE-1 occurs in at least two alternatively spliced forms, ECE-1α and ECE-1β, that differ in their N-terminal putative cytoplasmic regions (13–16). ECE-2 is a separate gene product with a sequence homology to ECE-1 of 59% and an acidic pH optimum of 5.5 (17). Recently, ECE-3 has been isolated and found to predominantly convert bigET-3 (18). In most tissues, ECE-1 appears to be the most abundant form of ECE (17).

Only few data are available to date regarding the regulation of ECE activity. For example, addition of ET-1 to pulmonary endothelial cell cultures has been observed to reduce both ECE-1 mRNA and protein, and this effect was inhibited by the ET_B antagonist BQ788, suggesting a role for the ET_B receptor in the regulation of ECE activity (19).

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Abbreviations used: ECE, endothelin converting enzyme; ET, endothelin; ET_A, endothelin A receptor; ET_B, endothelin B receptor; GFAP, glial fibrillary acidic protein; ir, immunoreactive; IEP, isoelectric point; K_i, inhibitory constant; K_m, Michaelis Menton constant; PBS, phosphate buffered saline; RT PCR, reverse transcriptase polymerase chain reaction; [*S*], substrate concentration; SDS-PAGE, sodium-dodecylsulfate polyacrylamide gel electrophoresis; V_{max}, maximal initial substrate hydrolysis rate (velocity); *v*, substrate hydrolysis rate.

0006-291X/99 $30.00

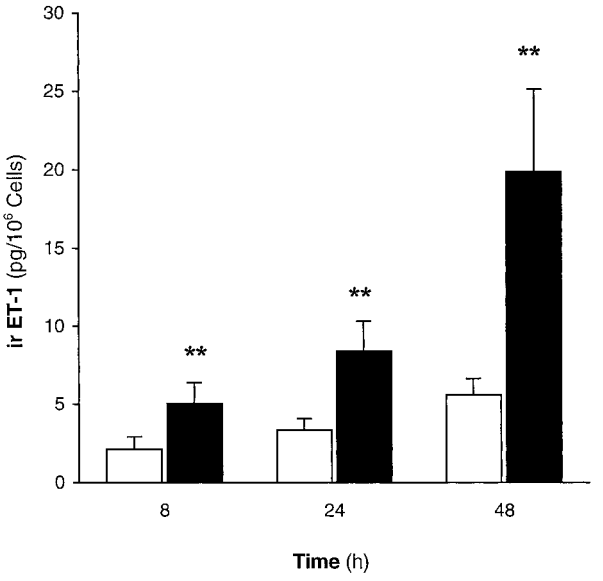

FIG. 1. Immunoreactive (ir) ET-1 concentrations over time in the supernatants of primary rat astrocyte cultures from wildtype (+/+; white bars) versus ET_B-deficient (sl/sl; grey bars) rats. $^{**}p < 0.01$ as compared to wildtype; $n = 5$.

In the brain, astrocytes express most components of the endothelin system, i.e. peptides and receptors, suggesting endothelin autoregulatory mechanisms in these cells (6, 20–22). Astrocytes fulfill endothelin eliminatory tasks intracerebrally, most likely via interaction of both ET_A and ET_B receptors (23). ECE activity has been described in membranes of astrocytes (24) which also express ECE-1 mRNA (25). The presence of ECE in astrocytes suggests an integrative role of this enzyme within the astrocytic endothelin system. Specifically, the question arises whether and how components of the astrocytic endothelin system influence ECE and vice versa. In previous experiments we found significantly higher ET-1 concentrations in astrocyte media from ET_B-deficient (sl/sl) versus wildtype rats (+/+), suggesting that this increase in basal ET-1 levels in the absence of functional ET_B receptors might be due to augmented synthesis of bigET-1, compromised reuptake/elimination of ET-1, and/or to an enhanced conversion of bigET-1 to ET-1 (25). Therefore, the present study has been designed to compare ECE expression and activity in astrocyte cultures from ET_B-deficient (sl/sl) versus wildtype (+/+) rats. We provide for the first time evidence that the ET_B receptor participates in the regulation of astrocytic ECE activity.

MATERIALS AND METHODS

Rats. For all experiments, animals from the Wistar-Imamichi AR strain (congenital aganglionosis rat) were used (26). These rats have an autosomal recessive 301 base pair deletion in the ET_B gene, spanning exon 1 and intron 1, corresponding to the first and the second transmembrane domains of the ET_B receptor (27). The animals were bred in our laboratory by mating of heterozygous sl/+ rats. The genotype of each animal used for experiments was confirmed by genotyping using PCR amplification of genomic DNA (25).

Cell culture. Primary astrocytes were obtained from cortices of 1-day-old Wistar-Imamichi rats, characterized by genotyping, and cultured in neuron-free conditions, as described previously (21). Cells were grown to confluence in plastic petri dishes (Falcon, Becton Dickinson, Heidelberg, Germany) in glutamine-free Dulbecco's modified Eagle's medium (Serva, Heidelberg, Germany), containing penicillin (50 U/ml) and streptomycin (50 μg), and supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany). The medium was exchanged twice weekly. Cells were used for experiments after 2 weeks in culture. The purity of astrocyte cultures was routinely determined by immunofluorescence using a mouse monoclonal antibody against glial fibrillary acidic protein (Boehringer, Mannheim, Germany), an astrocyte specific marker, with rhodamine-conjugated sheep anti-mouse antiserum (Boehringer) as secondary antibody. Subconfluent monolayers showed 98% positive staining for glial fibrillary acidic protein. For each experiment, only astrocytes from one preparation were used, and these cells (approximately $2–3 \times 10^6$ cells/dish) were switched into serum-free Dulbecco's modified Eagle's medium. Following the incubation times, conditioned media were harvested for peptide analysis, while the cells were used for membrane preparation, mRNA or protein isolation.

Extraction of peptides and radioimmunoassay. The astrocyte-conditioned medium was centrifuged ($2500 \times$ g, 10 min, 4°C) and concentrated by extraction through a Sep-Pak C18 cartridge (Waters Associates, Eschborn, Germany), pretreated with 60% acetonitrile/0.1% trifluoroacetic acid. The retained material was washed with 0.1% trifluoroacetic acid, eluted with 60% acetonitrile/0.1% trifluoroacetic acid, Speed-Vac-dried, and then exposed directly to an ET-1 specific radioimmunoassay (Peninsula, Belmont, CA, USA) with a detection limit of 1 pg/tube. Crossreactivities in this assay are 17% for bigET-1 and 7% for ET-3.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Whole-cell RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden,

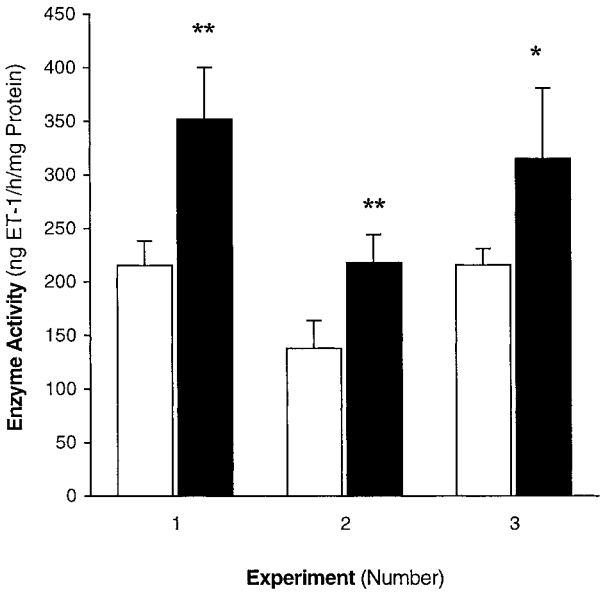

FIG. 2. ECE activity in membrane protein from wildtype (+/+; white bars) versus ET_B-deficient (sl/sl; grey bars) astrocytes. Three independent astrocyte preparations of both +/+ and sl/sl rats (experiments 1, 2, 3) have been analyzed. The mean ± standard deviation of six parallel determinations in each experiment is presented. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to wildtype.

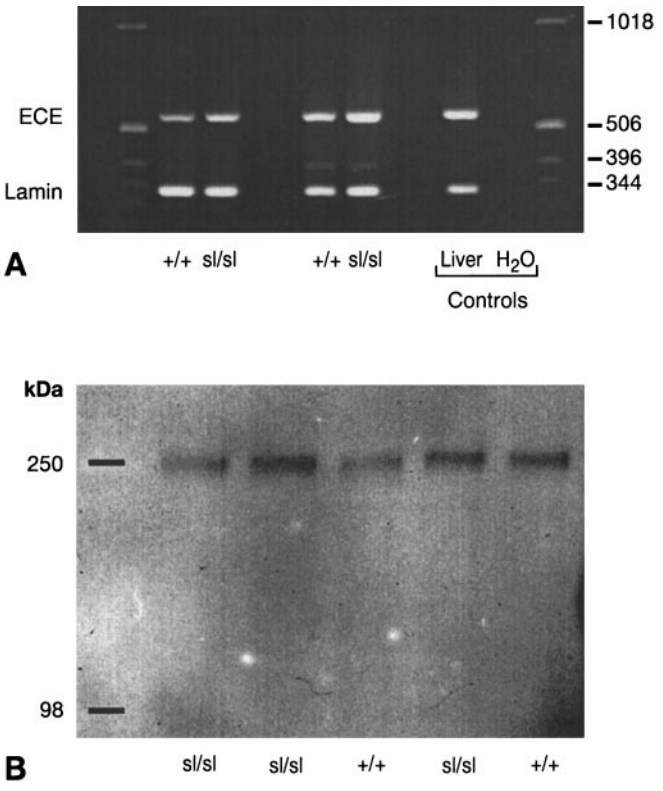

FIG. 3. (A) Analysis of ECE-1 gene transcription by RT PCR in primary astrocytes of ET_B-deficient (sl/sl) versus wildtype (+/+) rats. Specific mRNA expression under basal conditions is compared to that of the lamin-b PCR product (560 bp versus 335 bp). (B) ECE-1 protein expression shown by specific immunoblotting. 10 μg of membrane protein each from different preparations of +/+ or sl/sl astrocytes was subjected to SDS-PAGE and subsequent Western blotting. ECE-1 was detected with the specific polyclonal rabbit antiserum P6.

Germany). Total RNA (5 μg) was used to generate first strand cDNA by random priming with reagents and protocols used as recommended by the manufacturers (Amersham-Pharmacia, Freiburg, Germany; Gibco-Life Technologies, Karlsruhe, Germany). The cDNA representing 50 ng input RNA was amplified by PCR using Taq polymerase (Gibco) in a reaction volume of 50 μl. A specific primer pair was constructed from the reported rat gene sequence for ECE-1 (7) (CGC AAG TGT ACT ACC GTG CG and AGA CTC ATT GAT CTC CAC TG; expected PCR product: 560 bp). As stably expressed reference gene ("housekeeping gene"), rat lamin-b was used (23) (ATT GAG TAT GAG TAC AAG CTG and CGC ATC TCT CTC TCT TTG TC; expected PCR product: 335 bp). Both primer pairs were added simultaneously to the PCR reaction vials. Each primer pair amplified a single band of the expected size.

Measurement of ECE-1 isoform activity. Primary astrocytes (2–3 $\times$ 10^6 cells) were resuspended in 500 μl of buffer A (10 mM Tris, 5 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 20 μM trans-Epoxysuccinyl-L-Leucylamido(4-Guanidino)-Butane (E64), 20 μM leupeptin, 20 μM pepstatin, pH 7.0) and sonicated for 10 s at 0°C. The cell homogenates were centrifuged twice at 20 000 g for 20 min each, and the pellets resuspended in 500 μl of buffer A. 2.5 μg of membrane protein was incubated in buffer B (50 mM Tris/HCl, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) NaN_3, pH 7.0) with various concentrations of bigET-1 in a total volume of 160 μl for 30 to 60 min at 37°C. The enzymatic reaction was stopped by the addition of 10 μl of 150 mM EDTA pH 7.0. To measure the enzyme reaction constants V_{max} and K_m, 3.0 μg cell membrane protein was incubated in the presence of 0.09 to 1.8 μM big-ET-1. The substrate hydrolysis rate (v) per mg of membrane protein (nmol ET^{-1} h^{-1} mg^{-1}) was calculated. The maximal substrate conversion under the conditions used was $\leq$5%. K_m values were calculated using the Hanes plot ([S] versus [S]/v) where [S] is the substrate concentration). V_{max} values were calculated using the Eadie Hofstee plot (v versus v/[S]). To determine the K_i values of phosphoramidon for the inhibition of the ECE-1, membrane preparations were incubated in the presence of 0, 0.5 and 3.0 μM phosphoramidon as described above. Data were plotted in Lineweaver Burk diagrams. The mode of inhibition of astrocytic ECE-1 activity by phosphoramidon was competitive. The K_i values were calculated from secondary plots of the slope of the respective Lineweaver Burk curves versus the concentration of phosphorami-

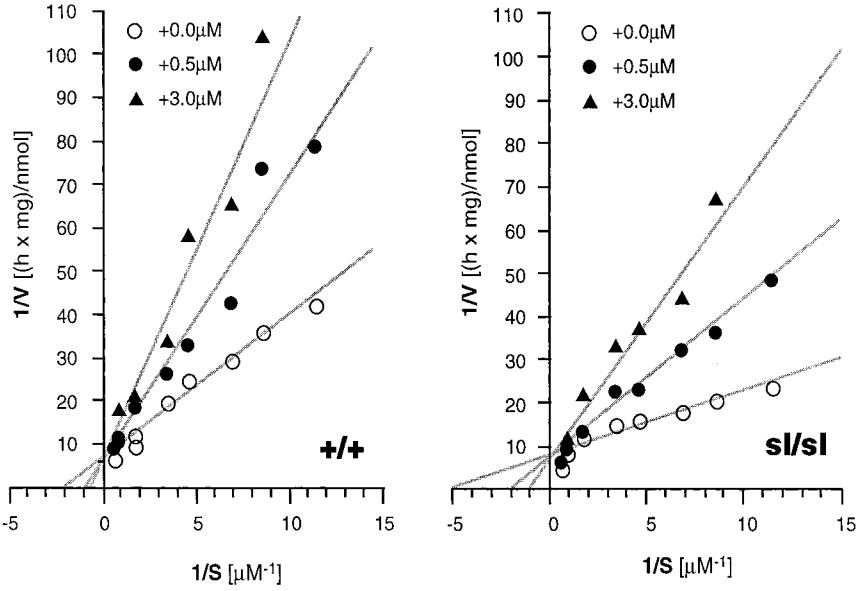

FIG. 4. Representative Lineweaver Burk diagrams of ECE-1 activity in +/+ and sl/sl astrocytes. ECE-1 activity was determined in the presence of 0, 0.5, and 3.0 μM phosphoramidon.

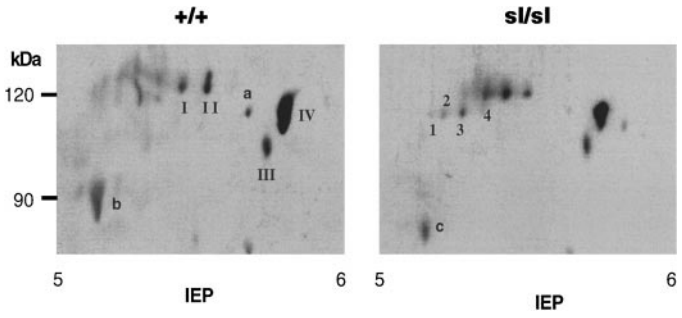

FIG. 5. Two-dimensional focussing of ECE-1 solubilized from +/+ and sl/sl astrocytes revealing heterogeneity with respect to charge and molecular weight (one of four independent experiments is presented). Four main isoforms I to IV with IEP values of 5.42, 5.51, 5.70, and 5.75 and respective molecular weights of 110 to 120 kDa are identical in +/+ and sl/sl cells. Four additional highly glycosylated isoforms 1 to 4 with IEP values of 5.17, 5.22, 5.28, and 5.36 are clearly detectable in sl/sl cells only. A glycosylation isoform with an IEP of 5.66 (a) is diminished in sl/sl cells. Both +/+ and sl/sl membrane preparations contain acidic glycosylation isoforms (b, c) with IEP values of 5.13 and 5.16 and molecular weights of 85 to 95 kDa.

don. The production of ET-1 in buffer B was measured using a specific radioimmunoassay as previously described (28). All kinetic experiments on cell membrane preparations were performed in triplicate. The hydrolysis of bigET was linear with time (up to 90 minutes) and protein (up to 20 μg) under the assay conditions used. The determination of produced ET-1 was also performed in triplicate.

Specific immunoblotting of ECE-1 from astrocytes. ECE-1 expression in astrocytes was determined by quantitative immunoblotting as previously described (15). In brief, 12 μg of cell membrane protein was heated to 55°C for 30 minutes in non-reducing sample buffer (Novex, Frankfurt am Main, Germany) and subjected to 4 to 20% sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; gradient gels, Novex) according to Laemmli (29). Electrophoresis was performed at 4°C with constant current of 25 mA/gel for 1.5 h. Proteins were transferred onto Immobilon-P membranes (Milipore) at 4°C for 2 h at 75 V constant voltage using 25 mM Tris, 192 mM glycine and 15% (v/v) methanol as a buffer system. See-Blue prestained standards (Novex) were used as molecular weight markers. The membranes were blocked for 18 h at 4°C, washed with phosphate buffered saline (PBS) supplemented with 0.2% (v/v) Tween-20 (PBS-T), incubated for 3 h at 20°C with antiserum P6 (1:500; v|v) and washed with PBS-T. The polyclonal antibody P6 recognizes an N-terminal peptide common to human and rat ECE-1. This antiserum enables specific detection of human or rat ECE-1 with low background, confirmed by preabsorption experiments using recombinant ECE-1 (15). ECE-1 was detected using the ECL-System (Amersham-Pharmacia) according to the manufacturer's instructions. ECL-hyperfilms were scanned and the ECE-1 signals were quantified using Scan Analysis version 2.50 (Biosoft, Cambridge, UK). Only the ECE-1 signal at 250 kDa was used for quantification.

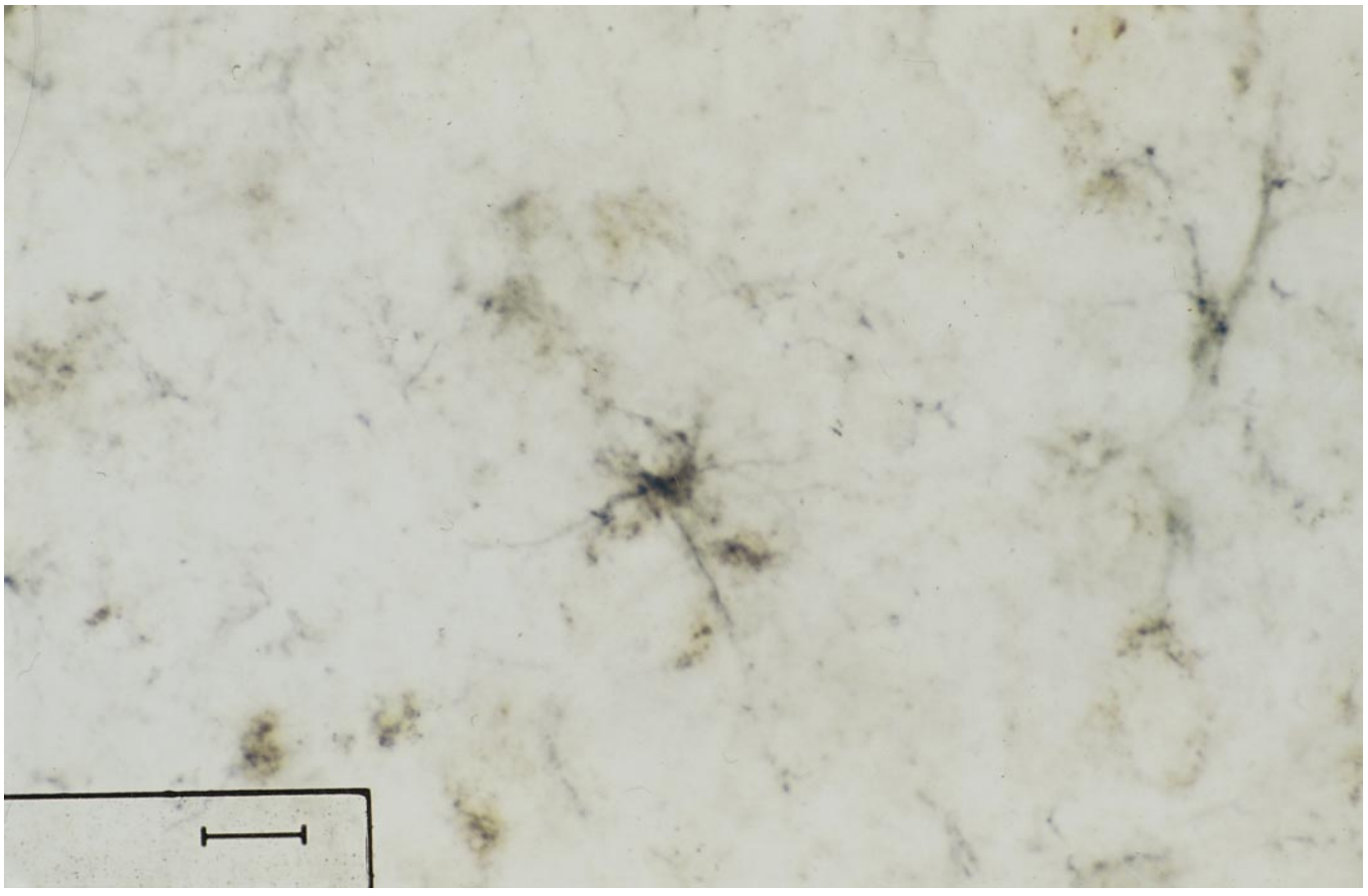

FIG. 6. Double-immunohistochemical documentation of astrocytic ECE-1 expression in the cortex of a +/+ rat 1 h after exposure to hypoxia (12 min, 2% O_2). Blue-grey reaction product: GFAP; brown reaction product: ECE-1a. Scale bar, 10 μm.

Protein was determined according to Lowry using bovine serum albumin as a standard protein (30).

Two-dimensional focussing of ECE-1. Crude membrane preparations of +/+ and sl/sl astrocytes were prepared as described. ECE-1 enzyme activity measurements and one dimensional SDS-PAGE followed by Western blot were performed. Protein was determined according to Lowry. Total membrane protein (0.5 to 0.7 mg) containing equivalent amounts of ECE-1 with respect to Western blot quantification was solubilized in sample buffer and subjected to isoelectric focussing on pH 4 to 7 gels as described (31). Isoelectric focussing gels were subsequently subjected to SDS-PAGE on 20 × 20 cm 7.5% gels. Polyacrylamid gels were blotted onto polyvinylidene difluoride membranes and immunostaining with polyclonal anti-ECE-1 rabbit antiserum was performed as described above.

Double-immunohistochemistry on rat brain sections. Three months-old +/+ and +/sl rats were exposed to 12 min of hypoxia (2% O_2) or to normal air (control) in a standardized chamber 60 min before being sacrificed. The brains were quickly removed and snap frozen. Cryostat cut brain sections (18 μm) from these rats were postfixed in 4% paraformaldehyde in PBS (30 min), permeabilized in 0.3% Triton-X/PBS (20 min), treated with 1% H_2O_2 (30 min), incubated with 10% blocking serum in a humid chamber (60 min; room temperature), and exposed to the first primary antibody for 24 h at +4°C, washed with PBS and incubated for 60 min with the appropriate biotinylated second antibody. Amplification of the signal was carried out by the avidin-biotin (ABC) method with the appropriate peroxidase kit (Vectastain Elite, Vector Laboratories Inc., Burlingame, CA, USA) and the first primary antibody signal visualized by incubation in 0.06% diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO, USA) and 0.006% H_2O_2 in 0.1 M TrisHCl (pH 7.6). The sections were then blocked with avidin-biotin blocking kit (Vector) and incubated with the second primary antibody using the above described protocol except that Vector-SG (Vector) resulting in a blue-gray reaction product was used as the chromogen for the second primary antibody. The slides were dehydrated, cleared with xylene, mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany) and coverslipped for investigation under a light microscope. Polyclonal rabbit anti-human ECE-1a antibodies (7.4 mg/ml) (32) and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibodies (0.02 mg/ml) (Boehringer Mannheim, Mannheim, Germany) were diluted 1:1000 with 2% blocking sera/PBS. Biotinylated goat anti-rabbit and horse anti-mouse second antibodies (Vector) were diluted 1:200 in PBS.

Statistical analysis. All values given in the text and figures are means ± standard deviation (SD) with n indicating the number of observations. Statistical analysis was done by unpaired two-tailed Student's t-test. A p value of <0.05 was considered significant.

RESULTS

Levels of ET-1 in culture media of primary rat astrocytes from ET_B-deficient versus wildtype rats (Fig. 1). Under basal conditions, immunoreactive (ir) ET-1 concentrations both in wildtype and sl/sl cultures increased over time. In ET_B-deficient astrocyte cultures, ir ET-1 levels were two- to four-fold higher as compared to those measured in +/+ cultures ($p < 0.01$ for all time points).

Cleavage of bigET-1 by ECE in wildtype (+/+) and ET_B-deficient (sl/sl) astrocytes (Fig. 2). To investigate whether the increased ir ET-1 levels in the media of ET_B-deficient astrocytes could be explained by an enhanced conversion of bigET-1 to ET-1, ECE activity was determined in astrocytic membrane preparations. Three independent cultures of both +/+ and sl/sl astrocytes, providing six parallel membrane preparations each, have been analyzed. ECE-1 activity was measured in 2.5 μg of membrane protein aliquots at a constant substrate concentration of 0.3 μM human bigET-1. The conversion of bigET-1 to mature ET-1 was significantly higher in sl/sl cells as compared to +/+ cells (Fig. 2). In the three subsequent experimental series, ECE-1 activity in sl/sl cells amounted to 156 ± 9% that of +/+ cells.

Reverse transcriptase polymerase chain reaction (RT PCR) and Western blot analysis of ECE-1 gene expression in astrocytes from ET_B-deficient (sl/sl) versus wildtype (+/+) rats (Fig. 3). In order to examine whether the altered astrocytic ECE activity, in the absence of functional ET_B receptors, would be due to an altered astrocytic ECE-1 RNA or protein expression, RT PCR as well as specific immunoblotting were performed. As demonstrated in Fig. 3A, there were comparable ECE-1 cDNA bands, relative to the lamin bands, in sl/sl as well as +/+ cells. Moreover, membrane preparations of +/+ and sl/sl astrocytes displayed comparable expression levels of ECE-1 protein. For thorough comparison, samples from +/+ and sl/sl cells were run in random order on the same SDS-gels. A total of 5 +/+ and 6 sl/sl membrane preparations were examined by SDS-PAGE, immunoblotting and subsequent scanning analysis. No significant difference in ECE-1 expression level was detected (Fig. 3B).

Kinetic analysis of ECE activity in primary astrocytes from ET_B-deficient (sl/sl) versus wildtype (+/+) rats (Fig. 4). Since RNA and protein levels of an enzyme do not necessarily reflect its catalytic activity, the enzymatic properties of ECE were analyzed kinetically. The apparent maximal initial specific velocity (app. V_{max}) of ET-1 production was comparable for ECE in +/+ and sl/sl cells (0.148 ± 0.008 and 0.132 ± 0.010 nmol $h^{-1}mg^{-1}$, respectively). The conversion of bigET-1 was competitively inhibited by phosphoramidon with K_i values of 0.6 and 0.3 μM. Representative Lineweaver Burk diagrams of ECE in +/+ and sl/sl cells are presented in Fig. 4. ECE from +/+ and sl/sl cells exhibited a significantly different K_m value: 0.51 ± 0.05 μM versus 0.20 ± 0.06 μM, respectively ($p < 0.05$). The quotient app. V_{max}/K_m was two-fold higher in sl/sl cells (183×10^{-6} $cm^3s^{-1}mg^{-1}$) as compared to +/+ cells (81×10^{-6} $cm^3s^{-1}mg^{-1}$), indicating an about two-fold increase in bigET-1 conversion rate in the former. To rule out the possibility that ECE activity measurement was affected by different concentrations of ET_A and (of course) ET_B receptors present on astrocyte membrane preparations, additional ECE assays were performed in the presence of the non-selective, non-peptidic endothelin receptor antagonist Bosentan. Preincubation of 3.0 μg of membrane protein of +/+

and sl/sl cells with 100 μM Bosentan for 30 minutes at 25°C did not significantly alter ET-1 production as compared to the respective controls (data not shown).

Two-dimensional focussing of ECE-1 solubilized from +/+ and sl/sl astrocytes. This procedure uncovered that ECE-1 showed heterogeneity with respect to charge and molecular weight. Four main isoforms I to IV (Fig. 5) with isoelectric point (IEP) values of 5.42, 5.51, 5.70 and 5.75 and respective molecular weights of 110 to 120 kDa were identical in +/+ and sl/sl cells. However, sl/sl cells showed four additional highly glycosylated isoforms 1 to 4 (Fig. 5) with IEP values of 5.17, 5.22, 5.28 and 5.36. A glycosylation isoform with an IEP of 5.66 (a, Fig. 5) is diminished in sl/sl cells. Both +/+ and sl/sl membrane preparations contain acidic glycosylation isoforms (b, c, Fig. 5) with IEP values of 5.13 and 5.16 and molecular weights of 85 to 95 kDa.

Double-immunohistochemistry on rat brain sections (Fig. 6). Our findings of a regulatory interaction between ECE and ET_B in astrocytes raised the question of whether astrocytic expression of ECE-1 might be detectable in vivo. Therefore, brains of adult +/sl and +/+ rats (sl/sl rats die of Hirschsprung's disease within the first month of life) have been screened by double-immunohistochemistry for GFAP and ECE-1. While under normal conditions no ECE-1 immunoreactivity could be detected in GFAP-positive cells (data not shown), brains from rats sacrificed 60 min after exposure to standardized hypoxia (2% O_2; 12 min) exhibited distinct astrocytic ECE-1 immunoreactivity (Fig. 6). No difference was detectable in the expression pattern between +/sl and +/+ rats (data not shown).

DISCUSSION

The present study demonstrates for the first time that ECE activity in astrocytes is modulated by ET_B receptors. Specifically, the conversion of bigETs into biologically active ETs is reduced in the presence of functional ET_B receptors. This is apparently achieved by ET_B mediated alterations in enzyme glycosylation which modifies substrate affinity.

The calculated molecular mass of the ECE-1 polypeptide chain is 87 kDa. The apparent molecular weight of about 250 kDa and 120 kDa of ECE-1 under non-reducing and reducing conditions has suggested that ECE-1 is highly glycosylated (9). The calculated IEP of the ECE-1 polypeptide, pH 5.63, is in agreement with the IEP determined in the present study for the two main ECE-1 isoforms III and IV both in +/+ and sl/sl astrocytes, indicating that glycosylation did not change the net charge of these isoforms. Furthermore, the 2D analysis strengthened our results from the one dimensional immunoblot analysis, indicating that more than 80% (+/+) and 90% (sl/sl) of total ECE-1 is highly glycosylated. The minor amount of unglycosylated ECE-1 present in the total membrane preparation used here most probably is unprocessed enzyme solubilized from the endoplasmic reticulum. Interestingly, +/+ and sl/sl cells differ with respect to the more acidic ECE-1 glycosylation isoforms. The addition of negative net charge is most likely due to the addition of N-acetylglucosamine residues to the protein. Recently, ECE-1 was found to bind to wheat germ lectin which specifically recognizes N-acetylglucosamine residues (33). This difference in glycosylation may account for the difference in ECE-1 activity observed comparing +/+ with sl/sl membrane preparations. We have demonstrated recently that N-glycosylation is essential for functional ECE-1 expression (12). This has also been shown for NEP 24.11 (11). In fact, the function of type II membrane proteins can be altered by domain-specific N-glycosylation (34). Our results suggest that this may also be the case for ECE-1 and, moreover, that the glycosylation pattern is regulated by ET_B receptors.

In the present study, neither ECE-1 mRNA nor protein were measurably influenced by the absence or presence of ET_B receptors in primary astrocytes under basal conditions. Thus, the observed N-glycosylation heterogeneity seems to be the only alteration of ECE-1 triggered by ET_B receptors. This is in some contrast to recent findings in rat pulmonary endothelial cell cultures demonstrating that addition of ET-1 reduced both ECE-1 mRNA and protein (19). Inhibition of this effect by the ET_B antagonist BQ788 suggested a role for the ET_B receptor in the regulation of ECE activity. This apparent controversy may be explained by differences among cell types as well as by differences in efficient ligand concentration. In fact, endogenous ET-1 under basal conditions may only lead to reduced ECE activity, whereas stimulated endogenous ET-1 or exogenously added ET-1, resulting in high levels, might provoke a downregulation of ECE-1 mRNA and protein. Few reports are available to date dealing with (patho)physiological modulation of ECE expression. For example, ECE-1 expression has been found to be increased in the kidneys of spontaneously hypertensive rats (35) and in the lungs of patients with idiopathic pulmonary fibrosis (36). It remains to be determined whether this increase could be related to alterations in ET_B expression under these conditions. To our knowledge, no reports exist on feedback modulation of ECE activity by posttranslational modifications within the enzyme protein.

Astrocytes in vitro, although in primary culture, have always to be considered as being somewhat activated even under "basal conditions" when compared to "resting astrocytes" in the brain. This may explain why, despite using comparable methods, expression of various components of the ET-system, peptides as well as receptors, can easily be identified under non-stimulated conditions in cultured astrocytes (6, 20–22)

but only weakly or not at all in astrocytes in situ (37, 38). Ongoing studies in our laboratory, using a standardized rat neurotrauma model, have revealed that astrocytic ET_B immunoreactivity is upregulated 48 h after injury, whereas it is hardly detectable in brains of sham treated animals (Sirén et al., submitted). Accordingly, ECE-1 expression in astrocytes in vivo could be identified in the present study only under stimulated conditions, i.e. hypoxia. Under such conditions, ET_B-mediated negative feedback regulation of ECE activity, and thus blunting of the production of mature ET-1, may be an important protective mechanism. These findings will have to be considered in ET-targeted therapeutic approaches to brain diseases.

ACKNOWLEDGMENTS

The authors thank Prof. H. Wiesinger, Tübingen, for critical review of the manuscript, and A. Eberle, H. Kamrowski-Kruck, M. Mangold and D. Roeder for skillful technical assistance.

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