Endothelin-1 protects astrocytes from hypoxic/ischemic injury

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ABSTRACT **Under pathological conditions such as ischemia (I), subarachnoid hemorrhage, and Alzheimer's disease, astrocytes show a large increase in endothelin (ET) -like immunoreactivity. However, it is not clear whether ET is protective or destructive to these cells during brain injury. Using astrocytes from ET-1-deficient mice, we determined the effect of ET-1 on these cells under normal, hypoxic (H), and hypoxic/ischemic (H/I) conditions. Under normal culture conditions, astrocytes from wild-type and ET-1-deficient mice showed no difference in their morphology and cell proliferation rates. ET-3 and ET_A receptor mRNAs were up-regulated whereas ET_B receptor mRNA was down-regulated in ET-1-deficient astrocytes, suggesting that ET-1 and ET-3 may complement each other's functions and that the expressions of these endothelins and their receptors are regulated by a complex feedback mechanism. Under H and H/I conditions, ET-1 peptide and mRNA were up-regulated in wild-type astrocytes, and the astrocytes without ET-1 died faster than the wild-type astrocytes, as indicated by greater efflux of lactate dehydrogenase. The present study suggests that astrocytes without ET-1 are more vulnerable to H and H/I injuries and that the up-regulation of astrocytic ET-1 is essential for the survival of astrocytes.—Ho, M. C. Y., Lo, A. C. Y., Kurihara, H., Yu, A. C. H., Chung, S. S. M., Chung, S. K. Endothelin-1 protects astrocytes from hypoxic/ischemic injury. *FASEB J.* 15, 618–626 (2001)**

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ENDOTHELIN (ET)-1, a 21-amino-acid peptide originally isolated from porcine aortic endothelial cells, is one of the most potent vasoconstrictors known (1). Three isopeptides—ET-1, ET-2, ET-3—have been identified (2). They exert various physiological actions by binding to two specific G-protein-coupled receptors subtypes, ET_A and ET_B receptors in mammalian cells (3, 4). ET_A receptor has greater affinity for ET-1 and ET-2 whereas ET_B receptor binds to ET-1, ET-2, and ET-3 equally well. Numerous reports on the expression, regulation, actions and bindings of ETs in various tissues other than endothelial cells have accumulated since the first isolation of ET-1 (5). Apart from being the strong vasoconstrictor (1, 6–8), ET-1 is known to be mitogenic (9–13) and anti-apoptotic (14, 15) in many cell types including endothelial cells and astrocytes in an autocrine and paracrine manner. Within the central nervous system (CNS), ET-1 is expressed in many neuronal groups (16–18) and in glial cells under pathological conditions (19). The distribution of ET-1 and its receptors, ET_A and ET_B, in different sites of the CNS suggests that it may influence a wide range of CNS controlled functions. Under pathological conditions of CNS such as stroke, hypoxic/ischemic (H/I) and infarction, subarachnoid hemorrhage, and Alzheimer's disease, ET-like immunoreactivity has been detected in astrocytes of brain (8, 20–24). Recently, we have shown that astrocytes in the damaged region of H/I mouse brain express a high level of ET-1 mRNA (25), indicating that increased endothelin-like immunoreactivity in the astrocytes is due to increased expression of ET-1 gene rather than increased binding of ET-1. These observations implicated a role of astrocytic ET-1 in the pathogenesis during H/I conditions. However, it is not clear whether ET-1 serves a protective role or hastens the death of the cells under H/I.

The ET-1-deficient mice have been generated in order to understand the physiological role of ET-1, but they die within minutes after birth due to craniofacial and cardiac deformities (26). It has been suggested that ET-1 is a growth promoting factor for neural crest cells and their derived tissues including branchial arches during early development, through its action on the ET_A receptor since ET-1 and ET_A receptor-deficient mice have similar defects in branchial arch derived tissues (27). This suggestion has been further supported by *in situ* hybridization analysis in which ET-1 mRNA has been shown to be transiently expressed in

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the branchial epithelium and mesodermal core between embryonic day 9.5 (E9.5d) and 10.5 (E10.5d) (28), whereas ET_A receptor mRNA was concurrently expressed in the neighboring mesenchymal cells (27).

To understand the significance of astrocytic ET-1 during normal, H, and H/I conditions, astrocytes were isolated and cultured from the embryos at embryonic day 17.5 (E17.5d) of wild-type ($ET\text{-}1^{+/+}$) and ET-1-deficient ($ET\text{-}1^{-/-}$) mice. Astrocytes deficient in ET-1 were found to be more vulnerable to the H and/or H/I conditions. We also found that ET-1 deficiency led to up-regulation of ET-3 and ET_A receptor and reduced expression of ET_B receptor mRNA. These observations suggest that ET-1 and ET-3 may complement each other's function and that the expression of these endothelins and their receptors is coordinately controlled.

MATERIALS AND METHODS

ET-1 knockout mice

ET-1-deficient mice were obtained from Dr. Kurihara (26). To collect E17.5d embryos of three different genotypes—wild-type ($ET\text{-}1^{+/+}$), heterozygous ($ET\text{-}1^{+/-}$), and homozygous ($ET\text{-}1^{-/-}$)—male and female $ET\text{-}1^{+/-}$ mice were mated. The genotypes of ET-1 knockout mice were determined by polymerase chain reaction (PCR) using genomic DNA isolated from tails. Forward primer (5′-ATC AGC AGC CTC TGT TCC-3′) from the region of the inserted *neo*^{r} gene and reverse primer (5′-CGT GGC CAG CCA TTG TAG AC-3′) from exon 5 of ET-1 gene were used to screened for the mutant allele. At the same time, another forward primer (5′-AAG CGC TGT TCC TGT TCT TCC-3′) from the region of exon 2 of ET-1 gene and the same reverse primer were used to screen for the normal allele. 30 cycles (94°C for 1 min, 59°C for 1 min, and 72°C for 1 min) were used to amplify for 1 kb product.

Astrocyte cultures

Primary cortical astrocytic cultures were prepared from the cerebral cortex of E17.5-d mouse embryos as described previously (29). In brief, the cerebral cortex freed of meninges was cut into small cubes in DMEM/F12. Cells were mechanically dissociated from the matrix by vortexing for 1 min and 30 s, then sieved through a 10 μm sterile nylon mesh filter. The cell suspension was subsequently plated in a T75 culture flask with normal growth medium, DMEM/F12 supplemented with 10% fetal calf serum (FCS). All cultures were incubated at 37°C with 95% air/5% CO_2. The cells were used for experiments after 4 wk. For the experiments that included astrocytes from both wild-type and ET-1 knockout mouse embryos, astrocytes of the same genotype were trypsinized, pooled, and seeded onto T75 culture flasks when the cultures reached confluency after 2 wk. Cells were used for experiments after incubation in the same conditions for 2 more weeks. The purity of the astrocytes was determined by immunocytochemistry using a rabbit anti-GFAP polyclonal antibodies (Dako, Carpinteria, Calif.) as mentioned below.

ET-1 ELISA assay

The astrocyte-conditioned media were collected at the indicated times. To determine the amounts of ET-1 in the media, the media were first concentrated by passing through C_2 columns (Amersham, Nutley, N.J.). The amount of ET-1 present in the media were detected by using an ET-1 ELISA system (Amersham).

Immunocytochemistry

Monolayers of astrocytes grown on glass coverslips in 24-well plates to ~90% confluence were fixed in 4% paraformaldehyde for 15 min at 25°C and postfixed with 95%/5% ethanol/acetic acid for 15 min at −20°C. Cells were then incubated for 24 h at 4°C with primary antibody, rabbit anti-GFAP polyclonal antibodies (Dako), diluted 1:200 in phosphate-buffered saline (PBS) with 5% normal goat serum. After subsequent washing in PBS, cells were reincubated with TRITC-labeled goat anti-rabbit antibody (Zymed Laboratories, San Francisco, Calif.), diluted 1:5000 in PBS for 1 h at 25°C. Control experiments without the primary antibody were done in parallel. Fluorescent images of the cells were obtained with a confocal microscope (Carl Zeiss, Thornwood, N.Y.).

Cell proliferation assay

Confluent astrocytes were trypsinized and seeded at a density of 1.7×10^4 cells/well in 96-well plates in normal growth culture medium. After 24 h, culture medium was replaced by serum-free medium, and cells were incubated for an additional 24 h. Astrocytes were then treated with 0.5% FCS or 10% FCS supplemented growth medium for 24 h. The number of viable cells in each well was estimated by measuring the rate of mitochondrial metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) using a cell proliferation assay kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions.

Hypoxia and hypoxia/ischemia

In vitro H and H/I culture conditions were performed as described previously (30, 31). All experiments were performed with an assay buffer containing 119 mM NaCl, 4.7 mM KCl, 1.0 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose at pH 7.4 and were filter sterilized. For all H experiments, the buffer for culturing astrocytes was first purged with N_2 for 30 min to replace the O_2 and then equilibrated with a gas mixture consisting of 5% CO_2/85% N_2/10% H_2 for 20 min in order to adjust the pH of the buffer. Astrocytes were then subjected to H for various times (4, 6, 8, and 24 h) in an anaerobic chamber (Forma Scientific, Marietta, Ohio) that preconditioned with 5% CO_2/85% N_2/10% H_2. The O_2 content in the chamber was measured by an oxygen electrode (Microelectrodes, Inc., Nashua, N.H.), and less than 10 ppm O_2 was detected. *In vitro* H/I was induced in astrocyte cultures by subjecting cultures to a combination of H and glucose-free conditions for various times (4, 6, 8, and 24 h). For some of the experiments, 10 nM ET-1 peptide (Peninsula Laboratories, Belmont, Calif.) was added to the medium before H/I treatment. The solvent for the peptide (100% acetonitrile with 0.1% trifluroacetic acid) was added to the medium of control cells.

Cell viability assay

Total cell viability was determined by the lactate dehydrogenase (LDH) assay (Boehringer Mannheim). At the indicated times, a small aliquot of the medium was removed from the culture flask and the amount of LDH was measured. Total cellular LDH was determined after adding Triton X-100 to a

final concentration of 1% in a T75 tissue culture flask. LDH released during the experiment was expressed as a percentage of total cellular LDH.

Reverse transcriptase PCR

Whole-cell RNA was isolated by TRI REAGENT® (Molecular Research Center, Cleveland, Ohio) according to the manufacturer's protocol. The conditions for reverse transcriptase (RT)-PCR have been optimized such that the amount of RNA added for RT reaction, the amount of reverse-transcribed cDNA, and the amount of primer pairs used for PCR as well as the number of amplification cycles were chosen within the linear range of the reaction. Total RNA (1.5 μg) after hybridization to 0.5 μg Oligo$(dT)_{18}$ was used to generate cDNA by Superscript™ II RNase H^- (Gibco BRL, Gaithersburg, Md.) with the respective synthesis buffer, 10 mM DTT, and 1 mM dNTP for 1 h at 42°C. The cDNA was then amplified by PCR using *Taq* polymerase in a reaction volume of 50 μl. Specific primer pairs were constructed based on the reported mouse sequences for ET-1 (CTT CCC AAT AAG GCC ACA GAC CAG/AGC CAC ACA GAT GGT CTT GCT AAG; expected PCR product: 426 bp), for ET-3 (GAT CAG AGA GGG GCT GTG AAG AG/AAG TAC AAC GCA AGC GTG TCC ACG; expected PCR product: 360 bp), for ET_A ET receptor (ACC GCC ATT GAA ATC GTC TCC ATC/TTA GCA GCA AGA AGC TGA GCA GTT C; expected PCR product: 445 bp), and for ET_B ET receptor (TGA CGC CAC CCA CTA AGA CCT CC/GCC TTC TGT ATG AAG GGC ACC AG; expected PCR product: 409 bp). For semi-quantitative analysis of the ET system gene expression, mouse GAPDH gene was coamplified in the same PCR reaction and used as the internal control to calculate the densitometric results. The primers used for amplifying GAPDH gene were TGA TGA CAT CAA GAA GGT GGT GAA G/TCC TTG GAG GCC ATG TAG GCC AT; expected PCR product, 239 bp. Both primer pairs were added simultaneously to the PCR reaction vials. After 4 min at 94°C, 29 to 30 cycles were performed at 94°C, 30 s; 61°C, 30 s; 72°C, 30 s. PCR products were examined on 1.75% agarose gel with ethidium bromide staining. Gel images were captured with the Eagle Sight system (Stratagene, San Diego, Calif.). The ratio of the ET system to GAPDH genes expression was determined by comparing the densities of bands for the ET system genes and for the GAPDH gene using PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Western blot analysis

Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.) and volumes of the sample used were adjusted to give equal loading to each lane. Proteins were separated by 7.5% polyacrylamide gel and transferred onto a nitrocellulose filter. Nonspecific sites were blocked by incubating with 5% nonfat dry milk powder in TBS buffer consisting of 0.05 M Tris, 0.15 M NaCl, pH 7.4 for 1 h. The nitrocellulose filter was then incubated at room temperature for 1 h with 1:200 dilution of mouse anti-GFAP antibody (Sigma, St. Louis, Mo.). Detection was performed with peroxidase-conjugated goat anti-mouse antibody with 1: 5000 dilution. Peroxidase activity was visualized using the ECL method (Amersham).

Statistical analysis

For semi-quantitative RT-PCR analysis, statistical significance was determined by paired *t* test using Graphpad prism (San Diego, Calif.). For all other experiments, statistical analysis was determined by one-way analysis of variance (ANOVA) and then Tukey's multiple comparison tests using Graphpad prism.

RESULTS

Increased ET-1 expression in wild-type primary cortical astrocytes under H/I conditions

Results from our previous experiments showed that astrocytes in the H/I damaged region of the mouse brain have a large increase in the level of ET-1 mRNA (25). To study the significance of astrocytic ET-1 during H and H/I conditions, we established primary cultures of astrocytes from wild-type embryos at E17.5d. The purity of the primary astrocytes was assessed by immunocytochemical staining using the polyclonal antibody specific for GFAP (see Fig. 2*B*). Approximately 95% of the cells were GFAP positive, suggesting that the normal culture conditions were optimum for maintaining astrocytes with high purity. As shown in **Fig. 1**, under H conditions, the amount of ET-1 secreted by the primary cortical astrocytes was increased after 24 h. Under H/I conditions, increased ET-1 secretion was evident as early as 4 h and with a much higher release at 8 h. Under normal conditions, there is no observable change in the ET-1 level. ET-1 level was not measured beyond 8 h of H/I because a significant number of cells were dead after that time. These results clearly indicated that *in vitro* H/I conditions closely resembles *in vivo* H/I conditions (25) and that an increase in ET-1 mRNA leads to increase of ET-1 peptide.

Characterization of normal and ET-1-deficient astrocytes in culture

We determined the phenotype of astrocytes from either wild-type or ET-1-deficient mice cultured under normal

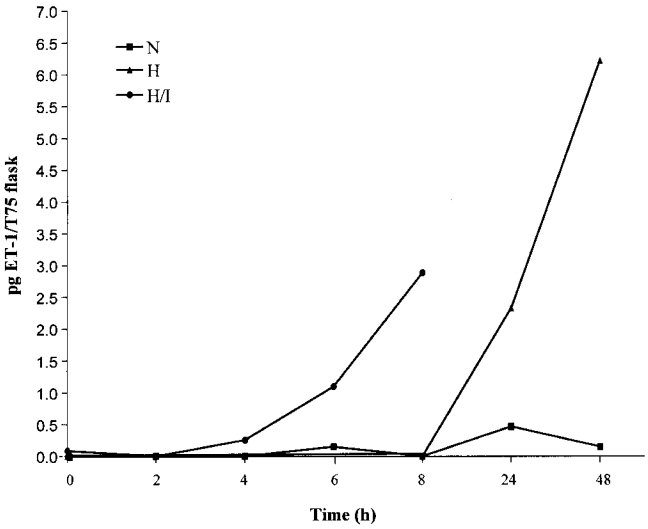

Figure 1. Amount of ET-1 peptide secreted by primary cortical astrocytes during various time points after normal (N), hypoxia (H), and hypoxia/ischemia (H/I) conditions.

conditions. As shown in Fig. 2*A, C*, under normal culture conditions, there was no significant morphological difference in astrocytes from wild-type and ET-1-deficient mice. Both cells expressed GFAP, characteristic of astrocytes, as determined by staining with antibodies against this protein (**Fig. 2*B, D***). The rate of cell proliferation was determined by MTT assays. As shown in **Fig. 3**, the growth rates of wild-type and ET-1-deficient astrocytes in normal (10% serum) as well as low serum (0.5% serum) media were indistinguishable.

Abnormal expression of ET-3 and ET_A and ET_B receptor in ET-1 deficient astrocytes

The expression of ET-1, ET-3 mRNAs as well as the mRNAs of their receptors in wild-type and ET-1-deficient astrocytes were further characterized to see whether there is any change in their expression to compensate for ET-1 deficiency. ET-3 is an ET isoform that is normally expressed in the brain. It differs from ET-1 by 6 of the 21 amino acids (2). These two endothelins are thought to share a number of functions (10, 32). Therefore, it is important to determine whether ET-3 is increased in the ET-1-deficient cells in order to compensate for the ET-1 deficiency. The levels of ET-3 mRNA in wild-type and ET-1-deficient astrocytes were compared by RT-PCR. The results, shown in **Fig. 4*B-2*** indicate that the level of ET-3 mRNA in ET-1-deficient astrocytes was ~2.5-fold higher than that of wild-type astrocytes, suggesting that ET-3 is overexpressed to compensate for the absence of ET-1. Levels of ET_A and ET_B receptor mRNAs, the two receptors for ET-1 and ET-3, were also examined by RT-PCR. Whereas ET_A receptor mRNA was increased in ET-1-deficient cells, ET_B receptor mRNA was lowered in these cells (Fig. 4*B-3, -4*).

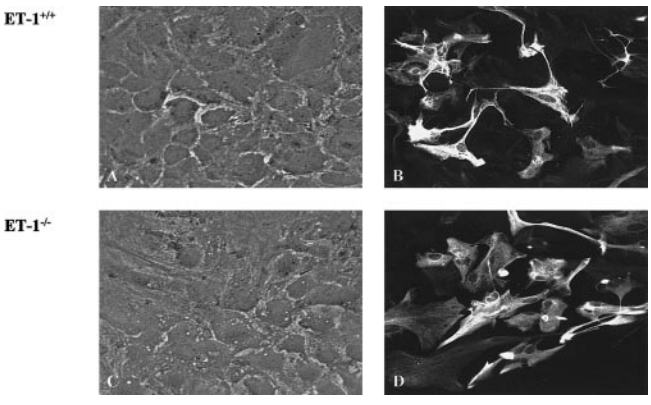

Figure 2. Micrographs illustrating the morphology (*A, C*) and GFAP immunostaining (*B, D*) of confluent astrocyte cultures from wild-type ($ET\text{-}1^{+/+}$) and ET-1-deficient ($ET\text{-}1^{-/-}$) mouse embryos. The morphology and intensity of glial fibrillary acidic protein (GFAP) staining are indistinguishable in $ET\text{-}1^{+/+}$and $ET\text{-}1^{-/-}$ astrocytes.

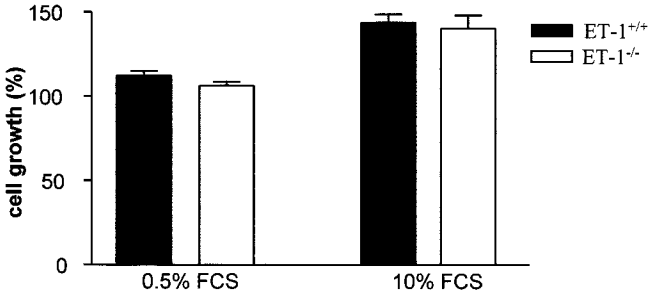

Figure 3. Histogram showing the rate of cell proliferation (MTT assay) in $ET\text{-}1^{+/+}$ and $ET\text{-}1^{-/-}$ astrocytes. The % of cell growth for $ET\text{-}1^{+/+}$ and $ET\text{-}1^{-/-}$ astrocytes in either 0.5% FCS or 10% FCS was not significantly different. Data are expressed as mean ± SE of 6 independent cultures.

ET-1-deficient astrocytes are more sensitive to hypoxic/ischemic stress

As mentioned above, *in vitro* H or H/I conditions clearly simulated brain H/I *in vivo* in that ET-1 synthesis was induced. To determine the effects of ET-1 secreted by astrocytes during H/I conditions, wild-type and ET-1-deficient astrocytes were cultured under H and H/I conditions. LDH released into the medium was used as measures of cell death. As shown in Fig. 4, ET-1-deficient astrocytes were much more sensitive to H/I stress. As early as 4 h after H/I, ~80% of the LDH activity in ET-1-deficient astrocyte culture was released into the medium, indicating that the plasma membranes of ~80% of these cells were leaky or that 80% of these cells were dying. In contrast, only ~30% of the LDH activity was detected in the medium of the wild-type astrocyte culture (**Fig. 5**) with induction of ET-1 synthesis (data not shown). By 8 h, all cellular LDH of ET-1-deficient astrocytes was released into the medium, whereas only ~40% of LDH activity was found in the medium of the wild-type astrocytes.

Regulation of ET-3 and ET_A and ET_B receptors during hypoxia and hypoxia/ischemia in normal and ET-1 deficient astrocytes

To further investigate the interacting regulation of endothelins and their receptors, the levels of ET-3 and ET_A receptors and ET_B receptor in wild-type and ET-1-deficient cells were determined under H and H/I conditions. As shown in **Fig. 6**, in wild-type astrocytes, H and H/I did not change the expression of ET-3 and ET_A receptor significantly (Fig. 6*B, C*). However, in ET-1-deficient cells, both ET-3 and ET_A receptor expression were significantly reduced under these conditions (Fig. 6*B, C*). There was a significant reduction of ET_B receptor level in wild-type astrocytes under H and H/I (Fig. 6*D*); a smaller reduction of this endothelin receptor was observed in ET-1-deficient astrocytes under H/I.

Effects of ET-1 treatment on hypoxic/ischemic astrocytes

To confirm that the increased sensitivity of ET-1-deficient astrocytes to H/I stress is due to the lack of

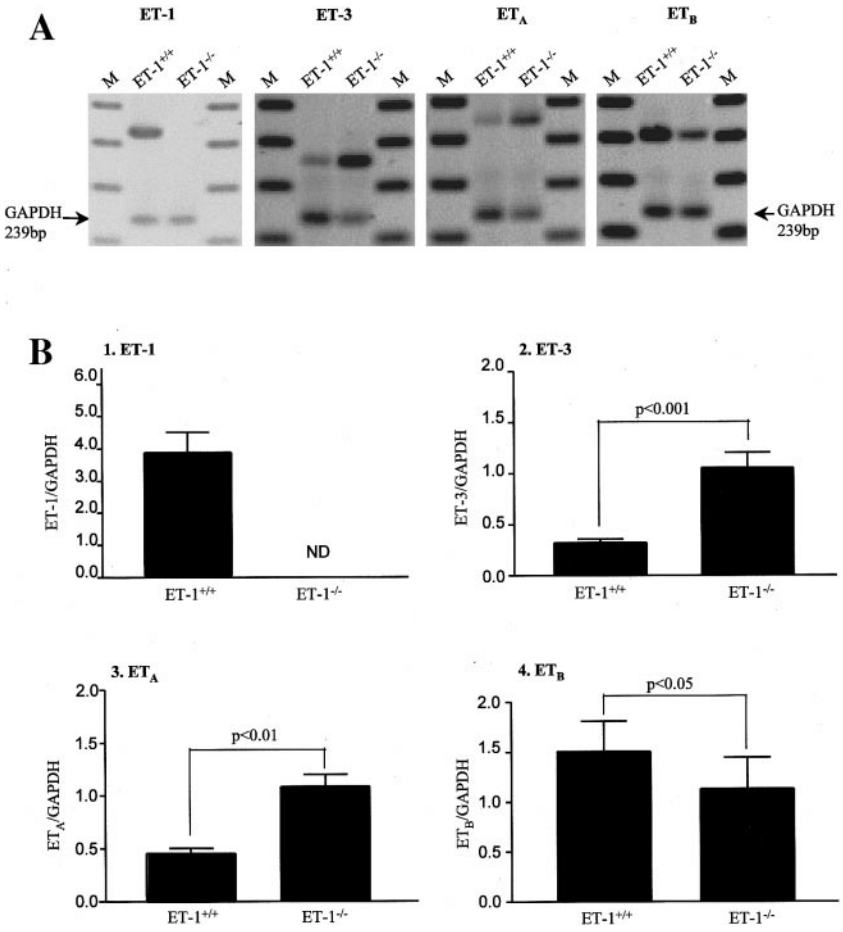

Figure 4. Semi-quantitative RT-PCR analysis of ET system gene expression in primary astrocytes of wild-type ($ET-1^{+/+}$) or ET-1 knockout ($ET-1^{-/-}$). *A*) Ethidium bromide-stained agarose gels of ET-1, ET-3, ET_A, and ET_B RT-PCR products. *B)* mRNA expressions of *1)* ET-1, *2)* ET-3, *3)* ET_A, and *4)* ET_B receptors under basal conditions were compared to that of GAPDH. The levels of gene expression were calculated as the ratios of band intensity of ET-related genes to that of GAPDH. Data are presented as mean ± SE, n = 5 to 7. Statistical differences between $ET-1^{+/+}$ and $ET-1^{-/-}$ astrocytes ($P<0.001$ for ET-3, $P<0.01$ for ET_A receptor, $P<0.05$ for ET_B receptor, one-way ANOVA and then Tukey's multiple comparison tests) are shown on the histogram. M-100 bp DNA ladder.

ET-1 and not to other defects generated during the process of knocking out ET-1 gene, ET-1 was added to the media of astrocytes under H/I to a final concentration of 1, 10, and 100 nM. As shown in **Fig. 7**, addition of 10 nM ET-1 partially normalized the LDH released by the ET-1-deficient astrocytes under H/I ($P<0.001$, one-way ANOVA and then Tukey's multiple comparison tests). However, adding ET-1 at a higher dose (100 nM) to the culture caused a significant rebound in the LDH level ($P<0.01$, one-way ANOVA and then Tukey's multiple comparison test). The amount of LDH released by the ET-1-deficient astrocytes cultured with exogenous 10 nM ET-1 was significantly lowered vs. those cultured with 1 nM also ($P<0.01$, one-way ANOVA and then Tukey's multiple comparison test).

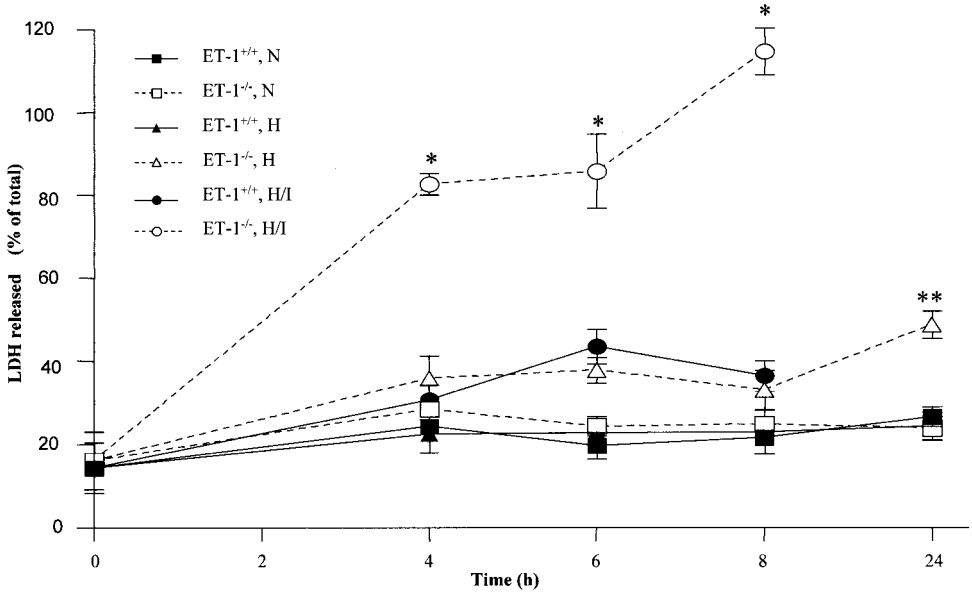

Figure 5. The % of lactate dehydrogenase (LDH) released by $ET-1^{+/+}$ and $ET-1^{-/-}$ astrocytes during normal conditions (N), hypoxia (H), and hypoxia/ischemia (H/I). Results are shown as mean ± SE of five different cultures. The % of LDH released was significantly different between $ET-1^{+/+}$ and $ET-1^{-/-}$astrocytes after 4, 6, 8 h H/I (*$P<0.001$, one-way ANOVA and then Tukey's multiple comparison test) and 24 h H (**$P<0.05$, one-way ANOVA and then Tukey's multiple comparison test).

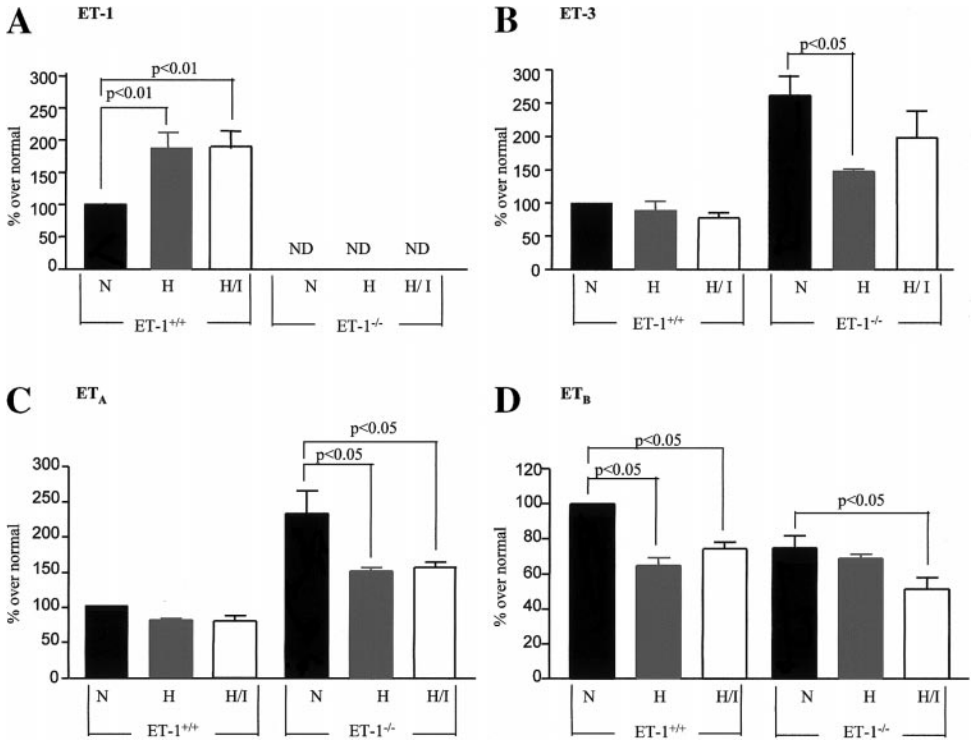

Figure 6. Semi-quantitative RT-PCR analysis of *A)* ET-1, *B)* ET-3, *C)* ET_A, and *D)* ET_B receptor mRNAs after 6 h hypoxia (H) and hypoxia/ischemia (H/I). The results, ET system gene expression/GAPDH ratio, were expressed as the percentage of values over that of normal. Data are presented as mean ± SE. $n = 3$ to 5. Statistical significance between different treatments to ET-$1^{+/+}$ or ET-$1^{-/-}$ astrocytes ($P<0.05$ or $P<0.01$, one-way ANOVA and then Tukey's multiple comparison test) are shown on the histogram.

Hypoxic/Ischemic induction of GFAP in normal and ET-1 deficient astrocytes

GFAP is the specific marker for astrocytes. The expression of this gene is induced upon H/I or other injuries. We therefore wanted to see whether ET-1 affects the expression of this gene in astrocytes under H/I. As shown in **Fig. 8**, the GFAP content was increased when wild-type astrocytes were cultured in H/I conditions. There was no increase in GFAP content in ET-1-deficient cells under H/I. However, the level of GFAP was induced only when exogenous ET-1 was added to the ET-1-deficient astrocytes under H/I conditions.

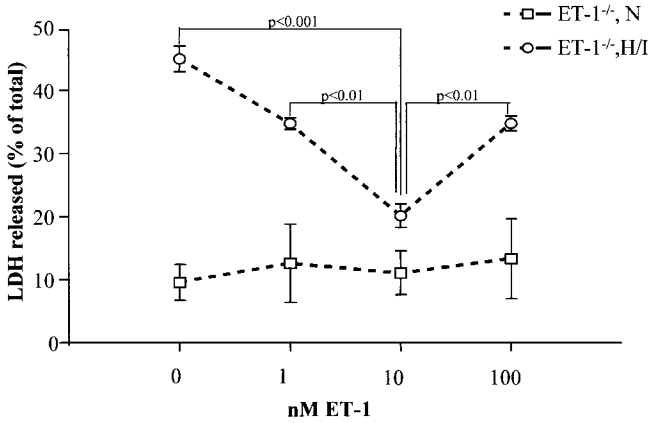

Figure 7. The % of LDH released into the medium by ET-$1^{-/-}$ astrocytes culturing in normal condition (N) and hypoxia/ischemia (H/I) for 6 h with ET-1 treatment. Data were expressed as mean ± SE of four to seven independent cultures. Note that there is a significant drop in the LDH release after addition of 10 nM ET-1 to the ET-$1^{-/-}$ astrocytes ($P<0.001$, one-way AVONA and then Tukey's multiple comparison test).

DISCUSSION

Astrocytes have many important functions in the brain because they are responsible for the maintenance of the neuronal cells nearby. They are involved in the glutamate uptake and therefore they may be responsible for preventing excitotoxic neuronal injury (33, 34). They regulate the levels of critical extracellular ions such as K^+ and H^+ (35) and are also responsible for the antioxidant defense in the brain (36–38). Under certain pathological conditions such as H/I and infarction, subarachnoid hemorrhage, and Alzheimer's disease, the astrocytes at the sites of degenerating neurons experiencing these stresses express ET-like immunoreactivity (8, 20–24). By *in situ* hybridization, we showed that H/I injury causes a dramatic increase in ET-1

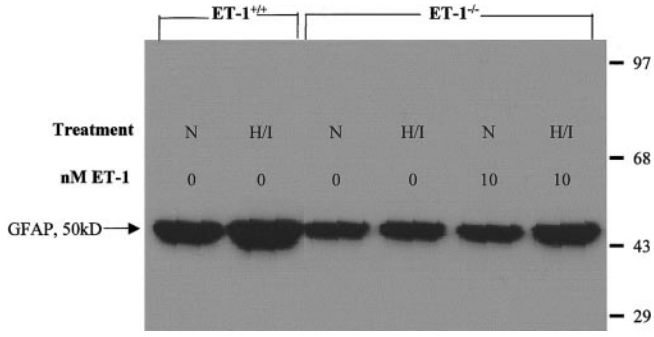

Figure 8. Western blotting of GFAP expression in ET-$1^{+/+}$ and ET-$1^{-/-}$ astrocytes culturing in normal (N) and hypoxia/ischemia (H/I) conditions for 6 h, with or without the addition of 10 nM ET-1. Note that the GFAP expression in ET-$1^{-/-}$ astrocytes was induced by the addition of 10 nM ET-1.

mRNA (25), indicating that increased ET immunoreactivity in the astrocytes of H/I brain is due primarily to increased synthesis of ET-1. All these observation suggest that the ETs may have an important function to response to these stresses. However, it is unclear whether ET-1 has a beneficial or a destructive function under these pathological conditions.

Although the ET-1-deficient mice are not viable (26), they provide a source of ET-1-deficient astrocytes for the analysis of ET-1 functions. We showed that under normal culture conditions, the ET-1-deficient astrocytes were almost indistinguishable from wild-type astrocytes in terms of morphology and growth rate. We do not know whether this is because ET-1 has no function in astrocytes under normal conditions or whether its functions under these conditions are taken over by ET-3, which is overexpressed in ET-1-deficient cells.

In the ET-1-deficient astrocytes, ET-3 and ET_A receptor mRNAs are increased whereas ET_B receptor mRNA is reduced. ET-3 is thought to have functions similar to ET-1. Its up-regulation in ET-1-deficient cells suggests that the expression of this gene may be mediated by feedback mechanism, possibly through their common receptors ET_A and ET_B. The increase in ET_A and a decrease in ET_B in the absence of ET-1 also suggest that the expression of these two genes may be regulated by some feedback mechanisms involving ET-1 under normal conditions. This feedback regulation of ETs and their receptors is also evident during H/I stress, as ET-3 and ET_A and ET_B receptor mRNAs are down-regulated in ET-1-deficient cells.

We cultured the astrocytes in the absence of oxygen and oxygen/glucose to simulate, respectively, H and H/I *in vivo*. We found that the *in vitro* H/I conditions elicited the induction of ET-1 synthesis in the astrocytes similar to *in vivo* H/I (25). Recently, hypoxia-inducible factor 1 binding site has been identified near the transcription start site of the ET-1 gene (39). Our present model may help to pave the way to increase our understanding of the mechanism of regulation of ET-1 synthesis by H/I conditions.

The specific induction of ET-1 during H/I suggests that ET-1 may have an important function to counteract this stress. We showed that ET-1-deficient astrocytes were much more sensitive to H/I than normal astrocytes, indicating that ET-1 has an important function of protecting the astrocytes against H/I stress. ET-1 and ET-3 are thought to exert similar effects on astrocytes with regard to binding to ET_B receptor, inositol phospholipid turnover, regulation of intracellular calcium, and mitogenesis (10, 32), suggesting that ET-1 and ET-3 can compensate for each others function including protection from cell death. However, even the increased level of ET-3 in the ET-1 deficient cells does not confer protection against H and H/I stress. This indicates that some functions of ET-1 cannot be replaced by ET-3 although both bind ET_B receptor.

Despite the possible protective effect of ET-1 on astrocytes during H and H/I, the ET_B receptor mRNA was found to be down-regulated by ~30–40% under these conditions. This is most likely in response to the large increase in ET-1 during H and H/I. As discussed later, too much ET-1 is toxic to the cells. Reducing ET_B receptor may reduce this toxicity. Also, the protective effect of ET-1 in the wild-type astrocytes may act through the ET_A receptor as the mRNA level of this receptor was not altered by H or H/I. It is possible that the ET_A receptor may partly take over ET_B's role when the ET_B receptor is down-regulated. As in astrocytes prepared from the ET_B receptor-deficient rat, the ET_A receptor becomes coupled to the $G_{i/o}$ protein, which was originally found to be involved in the ET_B receptor mediated protein kinase activation for mitogenic action of ET-1 and DNA synthesis in astrocytes (40).

Addition of ET-1 to ET-1-deficient astrocytes provided partial protection for these cells, supporting our notion that a lack of ET-1 made the cells more sensitive to H/I. The partial protection provided by exogenously added ET-1 probably reflects the very short half-life of this peptide *in vitro* (19). It is surprising to find that 100 nM ET-1 had a less protective effect against H/I than 10 nM ET-1. It is likely that at 100 nM ET-1, ET-1 has a toxic effect on the cells. This is supported by the fact that addition of 10 nM ET-1 to the wild-type astrocytes under H/I conditions actually led to more cell death and less activation of GFAP (data not shown).

The amount of LDH released from ET-1-deficient astrocytes in response to H/I shown in Fig. 7 was lower as compared to Fig. 5. The reason for this is not clear. The only difference was that the solvent for ET-1, acetonitrile and 0.1% trifluroacetic acid, was added to the media in these experiments. It is possible that these components may have blunted the cells' response to H/I stress.

Astrocytes are reactive to various injuries in brain as evidenced by increased expression of GFAP. ET-1 and ET-3 are thought to be responsible for activating the astrocytes, because injection of these ETs into the striatum of rat brain increased the number of GFAP-positive cells (43). In our *in vitro* H/I model, ET-1 appeared to be essential for the induction of GFAP in the astrocytes, but it is not clear whether ET-1 alone can induce the expression of GFAP. ET-3 alone clearly cannot activate astrocytes since there is lower level of GFAP in our ET-1-deficient astrocytes with ET-3 expression. Activation of astrocytes by the ETs is thought to be mediated via the ET_B receptor because BQ-788, a selective ET_B antagonist, can block this activation (43). It will be interesting to see whether this receptor antagonist has similar action *in vitro* because the cell culture system is more amenable to experimental manipulations.

Our results showed that induction of GFAP in the reactive astrocytes corresponded to resistance to H/I stress. Conversely, failure to induce GFAP expression in ET-1-deficient astrocytes is associated with susceptibility to H/I stress. Thus, the increase in GFAP content in astrocytes in response to H/I injuries in the brain indicates the brain's attempt to repair the damage. It appeared that ET-1 has an important function of pro-

tecting the astrocytes against H/I stress so that these cells can either repair their neighboring damaged neurons or participate in forming a protective boundary of the injured cells of the brain. FJ

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