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Endothelin 1 protects HN33 cells from serum deprivation-induced neuronal apoptosis through Ca^{2+}-PKCα-ERK pathway

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Abbreviations: ECL, enhanced chemiluminescence; EGFP, enhanced green fluorescent protein; ETs, endothelins; Fura-2AM, fura-2 acetoxymethyl ester; GPCR, G protein-coupled receptors; VIP, vasoactive intestinal peptide

Abstract

Endothelins (ETs), which were originally found to be potent vasoactive transmitters, were known to be implicated in nervous system, but the mode of mechanism remains unclear. ETs (ET-1, ET-2, and ET-3) were added to HN33 (mouse hippocampal neuron × neuroblastoma) cells. Among the three types of ET, only ET-1 increased the intracellular calcium levels in a PLC dependent manner with the induction of ERK 1/2 activation. As the result of ET-1 exposure, the survival rate of HN33 cells and the PKCα translocation into the plasma membrane were increased. We suggest that ET-1 participated in the neuroprotective effect involving the calcium-PKCα-ERK1/2 pathway.

Keywords: calcium; cell survival; endothelins; mitogen-activated protein kinase 1; neurons; protein kinase C

Introduction

The endothelin (ET) peptides are potent peripheral vasoconstrictors and composed of 21-amino acids (Yanagisawa *et al.*, 1988). ETs were initially believed to influence brain functions only indirectly through regulation of cerebral perfusion due to their vasoconstrictor activity. Recently, evidence has accumulated that ETs may fulfill a wider range of physiological actions within the CNS (Cintra *et al.*, 1989; MacCumber *et al.*, 1990; Kohzuki *et al.*, 1991). Within the CNS, ET-1 has been detected in the cerebral cortex, the hippocampus, amygdale, pituitary, hypothalamus and cerebellum (Lee *et al.*, 1990). Such diverse distribution suggests that ET-1 is involved in a wide range of brain functions. In some experimental studies, ETs bind to neurons and glia and stimulate phosphoinositol turn over (Lin *et al.*, 1989; Crawford *et al.*, 1990) and increase intracellular calcium concentrations (Holzwarth *et al.*, 1992). These findings support the idea of a functional role for ETs in the CNS.

The cellular and molecular mechanisms underlying the possible contribution of ETs to the neuronal process need to be further characterized. The aim of this study was, therefore, to obtain more insight into the molecular components involved in ETs-mediated signal pathway in the CNS.

Materials and Methods

Cell culture

HN33 cells (American Type Culture Collection, Rockville, MO), which were derived from the fusion of primary hippocampal neurons of postnatal day 21 mice and the N18TG2 neuroblastoma cell line, were bought. HN33 cells express a broad range of neuronal signaling properties (Watson *et al.*, 1994; Lenox *et al.*, 1996; Petitto *et al.*, 1998) and have been used to investigate pathophysiological features of neuronal injury states (Cunningham *et al.*, 1998; Shi *et al.*, 1998). In brief, HN33 cells were cultured as below (Shi *et al.*, 1998). They were maintained in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (HyClone Laboratories) plus 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C and 5% CO_2.

Treatment of cultured cells with ETs

The concentrations of ETs (Sigma-Aldrich, St. Louis, MO) were determined in preliminary experiments. ETs' effect on the calcium concentrations in HN33 cells were compared at 6 different ETs concentrations (100, 300, 900 nM and 1, 2, 3 μM). ET-1 at a concentration between 300 nM and 1 μM elicited concentration-dependent increase of intracellular calcium concentrations. According to this result, the concentration of saturation point 1 μM was chosen in this study.

Measurement of intracellular calcium concentration

The change of calcium concentration was measured using fura-2AM (Sigma-Aldrich). HN33 cells were incubated in serum-free RPMI 1640 medium with 3 μM fura-2/acetomethyl ester at 37°C for 30 min with continuous stirring. After washing with serum-free RPMI 1640 medium, the cells were suspended in serum-free RPMI containing 250 μM of sulfinpyrazone to prevent dye leakage. Approximately 2×10^6 cells were suspended in calcium-free Locke's solution (158.4 μM NaCl, 5.6 μM KCl, 1.2 μM $MgCl_2$, 5 μM HEPES, 10 μM glucose, 2.2 μM $CaCl_2$, 0.2 μM EGTA, pH 7.3) for each measurement. Changes in the fluorescence ration were measured with confocal fluorescence microscope at an emission wavelength of 500 nm for dual excitation wavelength at 340 and 380 nm. Calibration of the fluorescence ration versus calcium concentration was performed as previously described (Grynkiewicz *et al.*, 1985). For the signal pathways involved in calcium concentration, the cells were pre-treated with 2 μM of the specific PLC inhibitor U73122 (Calbiochem, EMD Biosciences Inc., San Diego, CA) for 30 min prior to the administration of ETs.

Western blot analysis of phosphorylated ERK1/2

The modulation of MAPK activity was investigated. The cells were grown in 6-well plates and at 60-70% confluence. The cells were serum-starved for 24 h before treatment at 37°C with the indicated agents. The media were then aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μl of lysis buffer (0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl and 50 mM Tris-HCl, pH 8.0) containing proteinase inhibitors (0.5 μM aprotinin, 1 μM PMSF, 1 μM leupeptin). The samples were then briefly sonicated, heated at 95°C for 5 min, and centrifuged for 5 min. Proteins were electrophoresed on 8% SDS-PAGE gel, and transferred to PVDF membranes. The blots were incubated overnight at 4°C with primary antibodies (anti-phosphoERK1/2 antibodies; New England Biolabs, Beverly, MA), and then washed 6 times with Tris-buffered saline/0.1% Tween 20 before probing with HRP-conjugated secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature. The blots were then visualized using enhanced chemiluminescence (ECL; Amersham Biosciences, Buckinghamshire, UK).

Morphological analysis and cell viability measurement

The morphological analysis and Western blots were performed via anti-apoptotic or pro-apoptotic Bcl-2 family members. Bcl-2 and Bax expression were analyzed by Western blots. Cell viability was assessed by Trypan blue exclusion. To evaluate cell proliferation, it was utilized the MTT assay, based on the conversion by mitochondrial dehydrogenases of the substrate containing a tetrazolium ring into blue formazan, detactable spectrophotometrically. The level of blue formazan was then used as an indirect index of cell density. Briefly, HN33 cells were seeded at a density of 5×10^4 cell/ml in 96-well plates, and then allowed to grow for 24 h. The growth media was replaced with serum free media for 24 h prior to dosing. The MTT reagents (7.5 mg/ml in PBS) was added to the cells (10 μl/well), and the culture was incubated for 30 min at 37°C. The reaction was then stopped via the addition of acidified triton buffer [0.1 M HCl, 10 % (v/v) Triton X-100; 50 μl/well], and the tetrazolium crystals were dissolved by 20 min of mixing on a plate shaker at room temperature. The samples were then measured on a plate reader (Bio-Rad 450) at a 595 nm test wavelength and a 650 nm reference wavelength. The effects of ETs on HN33 cells viability were assessed by MTT assay. In order to check downstream signal pathway, HN33 cells were pre-treated with 2 μM of the specific PLC inhibitor U73122 or 10 μM of the specific PKCα inhibitor Go (Calbiochem), 30 min prior to serum changes, and were then serum starved for 24 h. The results were representative of experiments repeated at least in triplicate.

Confocal imaging and immunoblotting analysis of PKCα translocation

The cells with enhanced green fluorescent protein (EGFP)-PKCα prior to ETs were transfected, and then observed the cellular location of PKCα. HN33 cells were then maintained in RPMI 1640 with 10% (v/v) FBS plus 50 U/ml penicillin, 50 μg/ml gentamycin sulfate and 50 μg/ml of streptomycin. The cells were plated on a culture-ware dish and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. DNA transfection was conducted with LipofectAMINE (Invitrogen, Carlsbad, CA). In brief, HN33 cells were seeded at 1×10^5 cells/6-well dish, then transfected by incubation with 1 μg of EGFP-PKCα (Calbiochem), and 6 μl of LipofectAMINE mixture, according to the instructions of the manufacturer.

Immunoblotting was performed by using anti-PKCα antibody. Cells were seeded in 24-well plates (5×10^5 cells/well) and starved for 24 h, then 1 μM of ET-1, ET-2, and ET-3 were treated for 10 min. 20 μg of cell lysates were prepared and

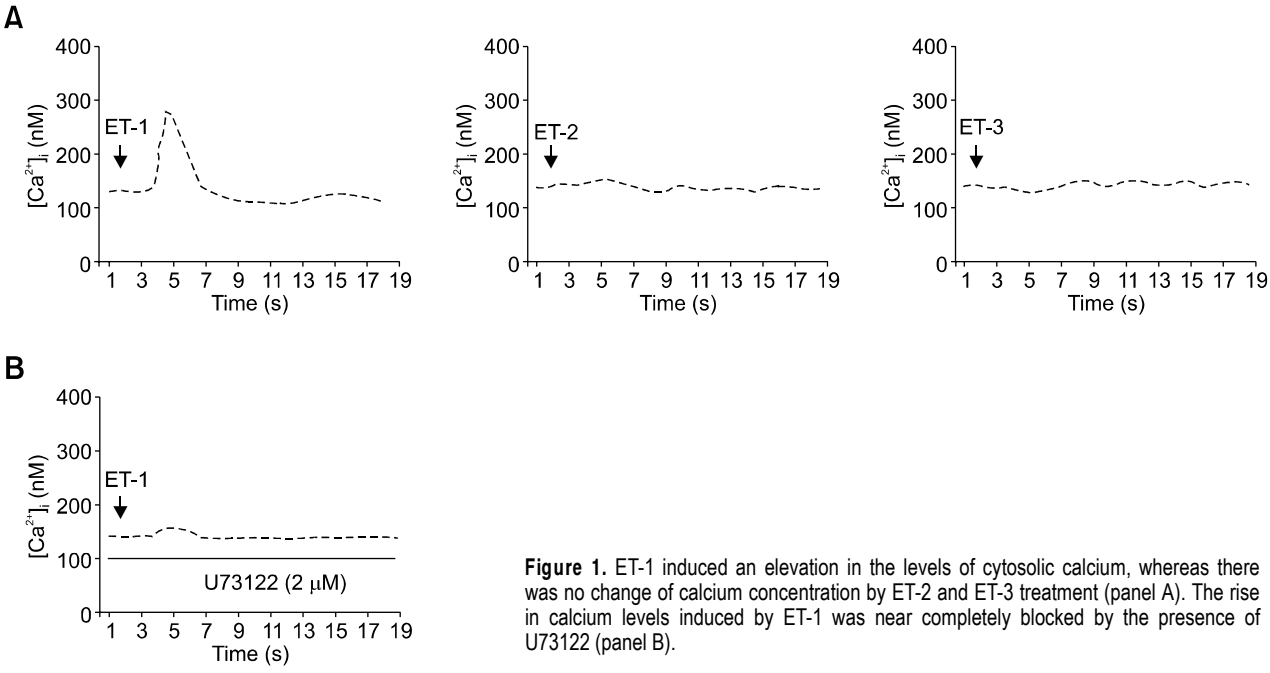

Figure 1. ET-1 induced an elevation in the levels of cytosolic calcium, whereas there was no change of calcium concentration by ET-2 and ET-3 treatment (panel A). The rise in calcium levels induced by ET-1 was near completely blocked by the presence of U73122 (panel B).

separated into plasma membrane part and cytosolic fraction. Representative Western blot experiment showed the effect of ET and the specific PKC activator, PMA (Calbiochem) on PKCα expression in lysate, plasma membrane and cytosol, respectively. Each fraction was subjected to SDS-PAGE, and then immunoblotted with anti-PKCα antibody.

Specific signal pathway of cellular survival effects of ETs

The cAMP-PKA activation was assayed by MTT. Vasoactive intestinal peptide (VIP) (Calbiochem, EMD Biosciences Inc.) was a well known AMP inducer and used as a positive control. HN33 cells were pre-treated with the specific PKA inhibitor H89 (Calbiochem), 30 min prior to serum changes, and were then serum starved for 24 h. The results were representative of experiments repeated at least in triplicate.

Statistical analysis

ANOVA and post-hoc test were used to compare the potency of various conditions. The mean values were considered to be statistically significant in cases in which the probability of the event was determined to be below 5% ($P < 0.05$).

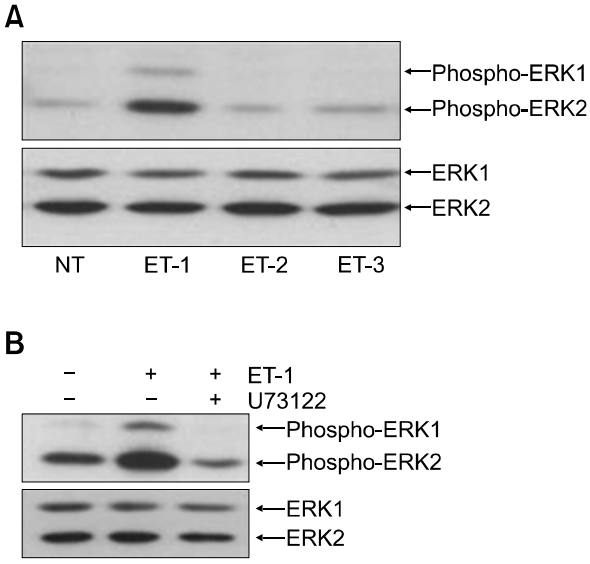

Figure 2. Immunoblotting with anti-phosphoERK1/2 and anti-ERK1/2 antibodies. The activity of ERK1/2 was noted to increase with ET-1 treatment, whereas ET-2 and ET-3 exerted no stimulatory effects (panel A). Pre-treatment with U73122 significantly suppressed the stimulatory effects of ET-1 on the activation of ERK1/2 (panel B).

Results

ET-1 induced an elevation in the levels of cytosolic calcium within 10 s. However, there was no change of calcium concentration by ET-2 and ET-3 treat-

ment. The rise in calcium levels induced by ET-1 was near completely blocked by the presence of U73122 (Figure 1).

The activity of ERK1/2 was noted to increase if ET-1 was treated, whereas ET-2 and ET-3 exerted no stimulatory effects on the activation of ERK1/2 (Figure 2A). Pre-treatment with U73122 was determined to significantly suppress the stimulatory effects of ET-1 on the activation of ERK1/2 (Figure 2B).

For the survival rate of serum free induced apoptosis, the rate increased with only ET-1 stimulation (Figure 3A). In the Western blotting (Figure 3B), the Bcl-2 protein was found and Bax protein was not expressed in cells pre-treated with ET-1. The effects of ETs on HN33 cell viability were assessed by MTT assay (Figure 3C). It was found there was survival effect after ET-1 treatment. But there were no survival effects after ET-2 or ET-3 treatments.

The cells treated with ET-1 exhibited translocation of PKCα to the plasma membrane (Figure 4A). Immunoblot analysis also confirmed the translocation of PKCα into the plasma membrane (Figure 4B).

There was no survival effects after ET-1 co-treatment in the presence of either U73122 or Go (Figure 5A). This suggests that both PLC and PKCα may be involved with ET-1's survival effects

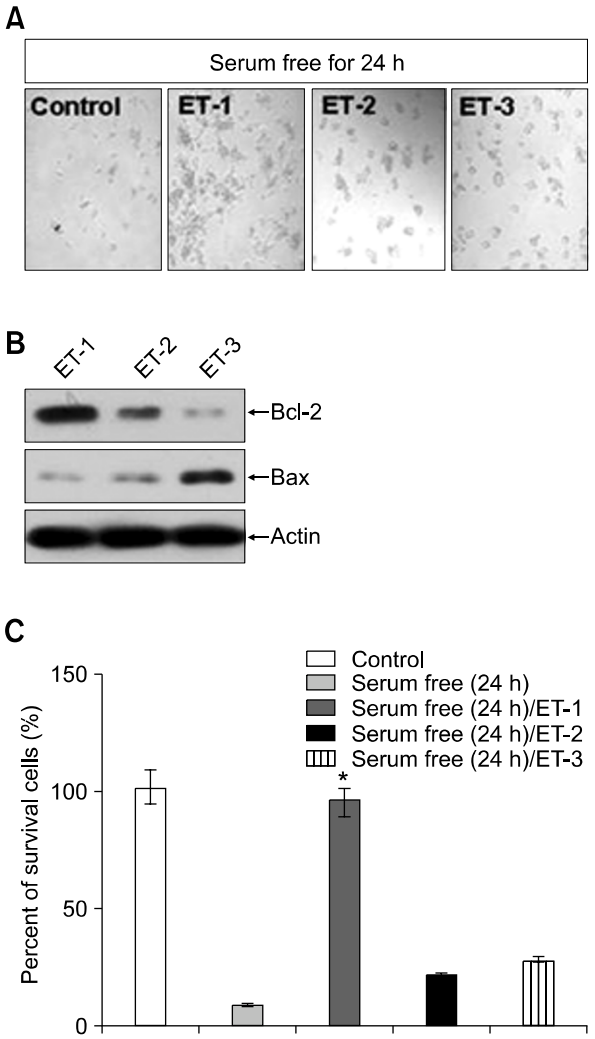

Figure 3. Morphological evidence for survival of HN33 cells with ET-1. HN33 cells with treatment of ET-1 were large and their processes were preserved, whereas cells with treatment of either ET-2 or ET-3 appeared shrunken and lacked processes (panel A). The Bcl-2 was expressed and Bax was not in HN33 cells with ET-1 pre-treatment (panel B). Effects of ETs on HN33 cell viability by MTT assay (panel C). *P < 0.05, as compared with control.

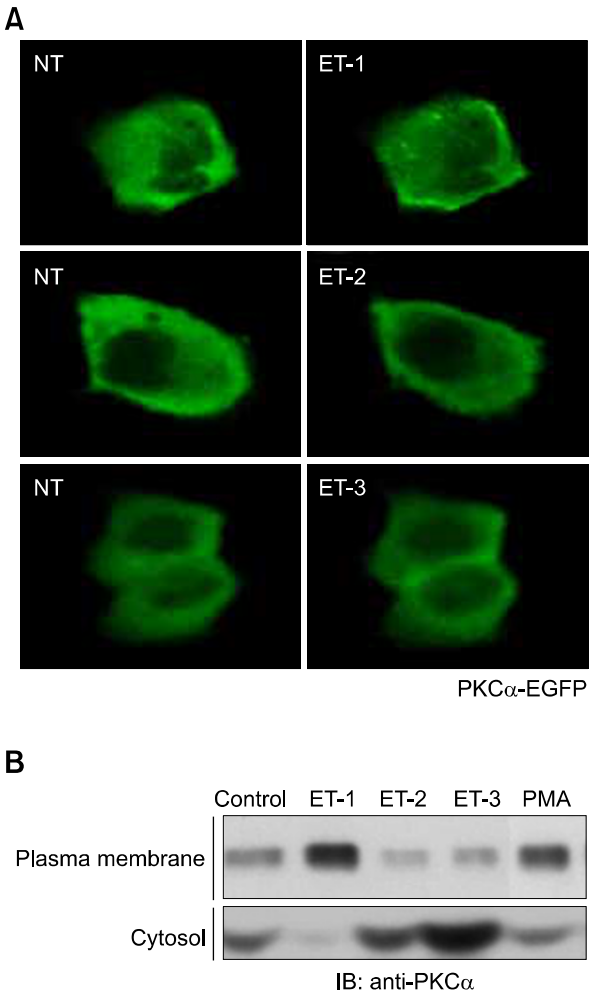

Figure 4. Effect of ETs on the translocation of PKCα. HN33 cells with ET-1 treatment exhibited translocation of PKCα to the plasma membrane, whereas cells with ET-2 or ET-3 did not exhibit (panel A). Immunoblotting (IB) was performed by using anti-PKCα antibody. HN33 cells with ET-1 treatment also confirmed the translocation of PKCα to plasma membrane, whereas cells with ET-2 or ET-3 did not (panel B).

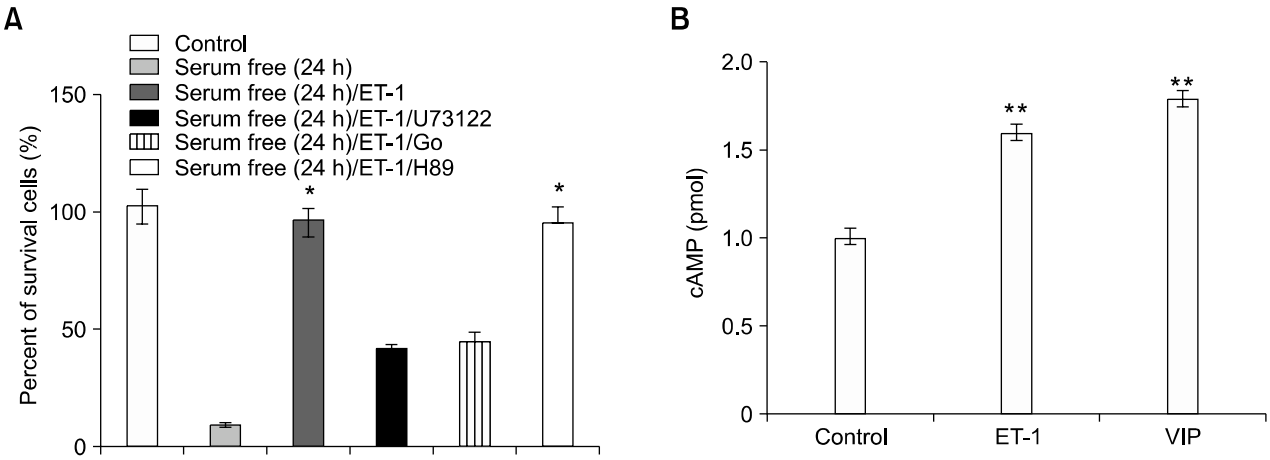

Figure 5. Cellular survival effects of ETs with/without U73122 and Go on HN33 cell viability by MTT assay (panel A). *P < 0.05, as compared with control. cAMP concentration was measured with ET-1 treatment and the AMP inducer VIP as positive control (panel B). **P < 0.05, as compared with control.

on HN33 cells. Next, regarding signal specificity issue, it was assessed whether ET-1 also stimulated the cAMP secretion (Figure 5B). This effect was not suppressed by the presence of the specific PKA inhibitor H89. Thus, these findings indicated that PLC-mediated calcium and PKCα activity might be involved specifically in ET-1-mediated neuron survival effects.

Discussion

ETs receptors have been cloned and identified as typical G protein-coupled receptors (GPCR) (Adachi *et al.*, 1991). GPCR regulates many signaling pathways such as MAPK or cAMP-PKA. This study showed that ET-1-mediated effects were through PKCα-ERK1/2 pathway. Although there might be other signal pathways such as cAMP-PKA, these neuroprotective effects on HN33 cells were not inhibited in the presence of the specific PKA inhibitor H89 in this study. These results suggest that ET-1 mediated neuroprotective effect may be due to calcium-mediated PKCα-MAPK pathways, rather than cAMP involvement.

The PKCα has been implicated in the cellular survival roles in nervous system (Pierchala *et al.*, 2004). This study showed that the activity of PKCα was increased further by treatment with ET-1, via the modulation of calcium levels. These findings indicated that ET-1 signaling probably merged with the MAPK signaling pathway, possibly via the conventional PKCα pathway. However, it was unable to test all of the possible pathways by which MAPK activation might occur, and so this study was unable to dismiss the possibility that other pathways might also be involved in the survival effects of HN33 cells.

This study suggested that such findings add a further piece of experimental evidence to the complex scenario of the cellular and molecular mechanisms underlying the pathogenic function of ET-1 in the CNS. Indeed, it is well known that the ET-1 and its receptor are expressed in various regions of brain (Rubanyi and Polokoff, 1994; Kuwaki *et al.*, 1999; van den Buuse and Webber, 2000). This suggested that ET-1 might be implicated in the pathogenesis of CNS disorders. Following various types of brain injury, alterations in ET-1 synthesis occur. For example, the ET-1 concentration in the cerebrospinal fluid is elevated in stroke patients, as well as in patients with subarachnoid hemorrhage (Lampl *et al.*, 1997). Similarly, ischemia or trauma in experimental animals results in an elevation of ET-1 within the CNS (Petrov *et al.*, 2002). These studies indicate that each ET might have specific function in the brain.

In conclusion, this study showed that ET-1 had effect on the HN33 cells via the calcium-PKCα-ERK1/2 pathway. Thus, these results not only constitute direct evidence of the role of ET-1 in HN33 cells, but also suggest that PKCα-ERK1/2 pathway participates in the ET-1-mediated neuronal survival. Therefore, here these results suggest a novel mechanism to explain the manner in which ET-1 modulates neuron functions. Of course, many aspects still remain to be further investigated. The molecular mechanisms underlying the above-mentioned ET-1 effects probably contribute to the neuronal survival and degeneration conditions, thereby representing potential pharmacological targets for treatment of neuro-degenerative disorders or neurotoxicity-related diseases.

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