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Brief Communication

Expression of Beta-catenin-interacting Protein 1 (CTNNBIP1) Gene Is Increased under Hypothermia but Decreased under Additional Ischemia Conditions

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It has recently been shown that hypothermia treatment improves brain ischemia injury and is being increasingly considered by many clinicians. However, the precise roles of hypothermia for brain ischemia are not yet clear. In the present study we demonstrated firstly that hypothermia induced beta-catenin-interacting protein 1 (CTNNBIP1) gene expression and its expression was dramatically decreased under ischemic conditions. It was also demonstrated that hypothermia activated endoplasmic reticulum (ER) stress sensors especially both, the phosphorylation of eIF2α, and ATF6 proteolytic cleavage. However, the factors of apoptosis and autophagy were not associated with hypothermia. These findings suggested that hypothermia controlled CTNNBIP1 gene expression under ischemia, which may provide a clue to the development of treatments and diagnostic methods for brain ischemia.

Key Words: Hypothermia, Ischemia, CTNNBIP1, ER stress sensor

Brain ischemia is also known as ischemic stroke, cerebral ischemia and cerebrovascular ischemia. Its main cause is insufficient blood flow to the brain. This results in the death of brain tissue by poor oxygen/ATP supply, which eventually leads to alterations in brain metabolism, reduction in metabolic rates, and energy crisis (Kristián, 2004; Kalogeris et al., 2014). Limited thrombolytics to prevent clots in the blood, caused by operative procedure of carotid endarterectomy may be used in parallel for brain ischemia treatment (Yong et al., 2013). However, thus far obvious cause and treatment effects have not been construed. It is known that hypothermia is one of the effective methods to reduce ischemic brain injury, and may be expected in emergency brain resuscitation of ischemic patients (Drury et al., 2014; Zhang et al., 2014; Lemmers et al., 2014). Cellular response of hypothermia results in altered gene expression and decreased oxygen/ATP consumption, however the detailed intracellular mechanism is unknown. Further study is required to delineate the precise mechanisms of how mild hypothermia prevents neuronal cell death. Our previous studies using the differential display (DD)-PCR experiment with Gene Fishing DEG premix kit (Seegene Co.), have shown that hypothermia increased the expression of beta-catenin-interacting protein 1 (CTNNBIP1) gene, a well-known negative regulator of the Wnt signaling pathway (Tago et al., 2000). Recent studies have illustrated the relationship between the hypothermia and intracellular signaling. Here, we tested whether the expression of

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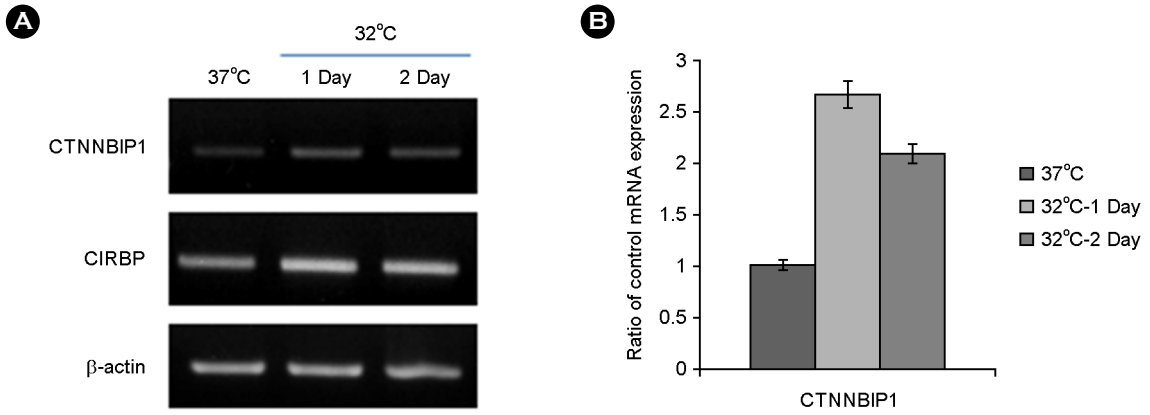

Fig. 1. Hypothermia increases CTNNBIP1 mRNA expression. PC12 cells were incubated under control conditions (37℃) or hypothermia (32℃) for 1~2 day. Hypothermia response was confirmed by increased expression of CIRBP mRNA which is a positive marker. CTNNBIP1; beta-catenin-interacting protein 1, CIRBP; cold-induced RNA-binding protein. The experiments were performed thrice and the results represent the average (B) and representative results are shown (A).

CTNNBIP1 was associated with hypothermia, ischemia, endoplasmic reticulum (ER) stress, apoptosis and autophagy, especially ER stress signaling.

PC12 cells (derived from a pheochromocytoma of the rat adrenal medulla) were cultured on collagen coated flasks in 85% RPMI 1640 supplemented with 25 mM Hepes buffer, 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 g/l d-(+)-glucose, and antibiotics: 25 μg/ml streptomycin and 25 U/ml penicillin at 37℃ in a 5% CO_2 atmosphere. Total RNA from cultured cells was extracted using an RNA isolation reagent (TRI-Reagent Ambion, Austin, USA). RT-PCR using the forward primer F (5'-AGTGGTGGCCACTAATGGAG-3') and R (5'-TCTTTTGTCAGGGGTCGTTC-3') for Bip; F (5'-GGGAGTCTTGTCGTGGAATTG-3') and R (5'-TGCTTTCCAAGACGGCAGA-3') for calnexin; F (5'-CAGGATTTGCCCTATCCAGA-3') and R (5'-GTCATTCCGTTCCTTCTCCA-3') for PDI; F (5'-ATGAACCGTGAGGGAGCAC-3') and R (5'-GATCTGGAAAACGCCATCAGC-3') for CTNNBIP1; F (5'-TCAGCTTCGACACCAATGAG-3') and R (5'-GTATCCTCGGGACCGGTTAT-3') for CIRBP; F (5'-TTACCTCCACCAGCAGGAAC-3') and R (5'-ACCACCTCTCTGTGCAATCC-3') for Bak1; F (5'-AAGCTGCACAGCGGGGCTAF-3') and R (5'-CAGATGCCGGTTCAGGTACT-3') for Bcl2; F (5'-GCCTGTCCTGGATAAGACCA-3') and R (5'-GTTCACCAGCAGGAAGAAGG-3') for LC3; F (5'-GTGCTCCTGTGGAATGGAAT-3') and R (5'-GCTGCACACAGTCCAGAAAA-3') for Beclin; F (5'-ACATCAAATGGGGTGATGCT-3') and R (5'-AGGAGACAACCTGGTCCTCA-3') for β-actin were performed for 30 cycles [94℃ for 30 s; 58℃ for 30 s; and 72℃ for 1 min (but 10 min in the final cycle)] with *Taq* DNA polymerase. Western blotting detection system kit (Amersham, Uppsala, Sweden) was used for ATF6 fragmentation and phosphorylation of eIF2α. eIF2α-P antibody and goat anti-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-ATF6 antibody was obtained from Imgenex (San Diego, CA, USA).

PC12 cells were incubated under control conditions (37℃) or hypothermia (32℃) for 1 or 2 day, respectively. The expression of cold-induced RNA-binding protein (CIRBP) was used as a positive control (Al-Fageeh and Smales, 2009). We found that the expression of CTNNBIP1 was increased about 2.5 folds by 1 day-hypothermia compared to its control; but the exposure to 2 day-hypothermia induced a slight decrease (about 2 fold) in gene expression. The result showed that hypothermia increased CTNNBIP1 mRNA expression (Fig. 1). We surmised from the result shown in Fig. 1, that hypothermia obviously induced the expression of CTNNBIP1 gene. We determined CTNNBIP1 gene expression in both, cells treated with hypothermia

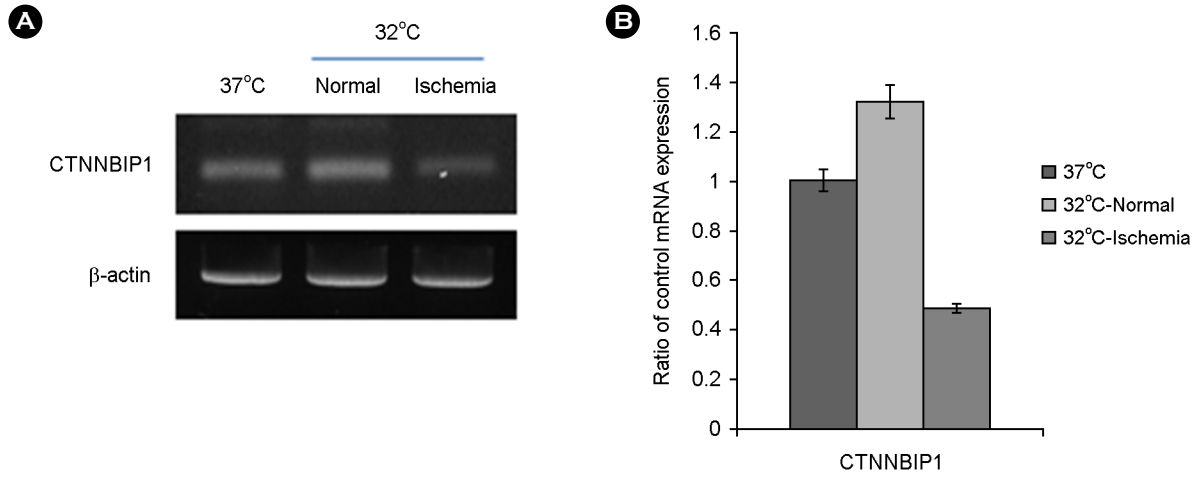

Fig. 2. Hypothermia decreases CTNNBIP1 mRNA expression under the ischemia. PC12 cells were incubated under control conditions (37℃), hypothermia (32℃), ischemia under hypothermia (32℃) for 1-2 day, respectively. CTNNBIP1; beta-catenin-interacting protein 1. The experiments were performed thrice and results represent the average (B) and representative findings are shown (A).

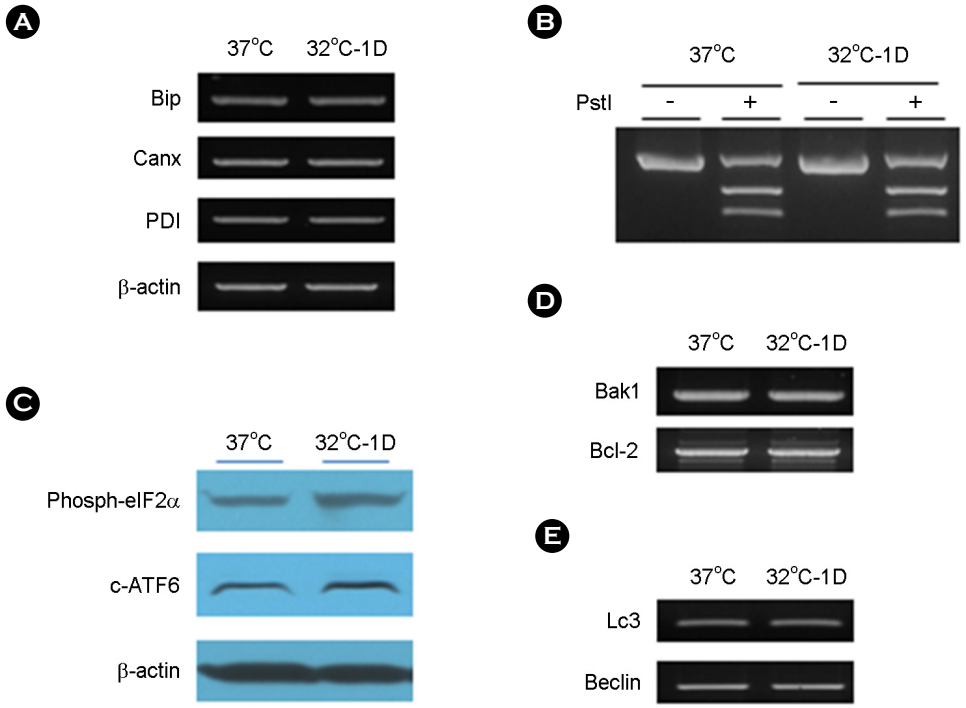

Fig. 3. Effect of hypothermia on gene expression of ER chaperones (A), XBP1 mRNA splicing (B), ER stress sensors (C), apoptosis-associated factors (D) and autophage-associated factors (E). PC12 cells were incubated under control conditions (37℃) or hypothermia (32℃) for 1 day. Gene expressions were determined by RT-PCR by conditions described in Materials and methods (A, D, E). The product of XBP1-Pst1 digestion was subjected to electrophoresis on the agarose gel (B). Both cleavage of ATF-6 and phosphorylation eIF2α were assessed by Western blotting (C). The experiments were performed thrice and results represent the average and representative findings are shown. Bip; binding immunoglobulin protein, Canx; calnexin, PDI; protein disulfide isomerase, ATF6; activating transcription factor 6, phosph-eIF2α; phosphorylation of eIF2α, Bak1; BCL2-antagonist/killer 1, Bcl-2; B-cell lymphoma 2, LC3; microtubule-associated protein 1 light chain 3α, beclin; coiled-coil myosin-like BCL2-interacting protein.

alone and cells treated with hypothermia together with ischemia, to verify whether hypothermia induced cells were related to ischemia. As shown in the Fig. 2, CTNNBIP1 gene expression significantly decreased to about 0.5 fold compared to its control. At this point there is no data to support that CTNNBIP1 gene expression is related to ischemia, or especially associated with hypothermia. However, the result shown in Fig. 2 demonstrated that hypothermia decreasingly modulated CTNNBIP1 mRNA expression under ischemia. We have tested whether hypothermia controlled ER chaperones (Bip, calnexin and PDI) (Braakman and Bulleid, 2011), the downstream-factors of ER stress sensors (XBP1, eIF2α and ATF6) (Lee and Ozcan, 2014), apoptosis-associated factors (Bak1 and Bcl-2) (Lovat et al., 2003) and autophage-associated factors (LC3 and beclin) (Jin and Klionsky, 2014). Cells exposed to hypothermia had no changes in ER chaperones gene expression (Fig. 3A), apoptosis-associated factors (Fig. 3D) and autophage-associated factors (Fig. 3E). Accumulation of un-/misfolded proteins in the ER lumen triggers an ER stress signal pathway through ER stress sensors. It is well known that, upon ER stress, releasing Bip from the ER luminal stress sensors cleaves ATF6α, XBP1 mRNA splicing and phosphorylation of eIF2α (Shore et al., 2011). Although no spliced XBP1 (Fig. 3B) was observed, both the cleavage of ATF-6 and phosphorylation eIF2α was clearly increased (Fig. 3C). The result of Fig. 3 showed that hypothermia controlled CTNNBIP1 mRNA expression through the activation of down-stream ER stress sensors, i.e. cleavage of ATF-6 and phosphorylation eIF2α, but not ER chaperones.

In summary, the present study was the first to demonstrate that hypothermia induced CTNNBIP1 mRNA. Additionally, its expression was further decreased to less than control levels under conditions of ischemia. CTNNBIP1 gene expression by hypothermia was associated with unfolded protein response (UPR); and activation of ER stress sensors, especially higher phosphorylation of eIF2α and ATF6 cleavage but unchanged ER chaperone expression. No alternative mRNA expression of apoptosis- and autophage-associated factors, was observed in cells treated with hypothermia. Our findings suggested that hypothermia decreased CTNNBIP1 gene expression under ischemia, which may provide new insight into the possibility of hypothermia treatment for ischemia, and may help in the development of novel methods for easy and quick diagnosis of neurodegenerative disorders, including ischemia and hypoxia.

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