INTRODUCTION

Many cellular processes including glucose metabolism, protein synthesis, cell survival, cell proliferation, cell migration, and neural plasticity are due to the involvement of Akt [1-3]. Akt (commonly known as protein kinase B, PKB) has also been seen to play a role in many types of neuroprotection including ischemic preconditioning [4-6]. Upon activation, Akt phosphorylates...
and inactivates glycogen synthase kinase-3β (GSK3β), which is involved in both caspase-independent and caspase-dependent mechanisms of cell death, consequently alleviating brain injury [7]. The survival and differentiation of developing neurons are supported by growth factors which act as upstream modulators of Akt. Furthermore, they protect neurons from atrophy and apoptosis [8, 9]. Thus, PI3K/Akt signaling is considered to be neuroprotective in neurodegenerative disorders. However, phosphorylation of Thr308 and Ser473 is required for full Akt activation via upstream signaling of kinases such as mammalian target of rapamycin (mTOR) complex 2, 3-phosphoinositide-dependent kinase-1 (PDK1), and DNA-dependent protein kinase [10, 11].

In glial cells, PI3K inhibition has been shown to lead to apoptosis in primary cortical astrocytes [12]. Furthermore, overexpression of a constitutively active GSK-3β is sufficient to cause astrocyte apoptosis and involves the inhibition of nuclear factor kappa B (NFκB). Previously, we also reported a possible signaling pathway from Akt/GSK3β to CREB in the astrocytes of excitotoxically damaged mouse hippocampi [13]. Furthermore, we reported Akt is activated by PDK1 in activated astrocytes [14]. Despite the well-established importance of Akt and cellular defense, it remains unclear how Akt is inhibited or suppressed. CTMP, first reported by Maira et al. [15], is an endogenous inhibitor of Akt that specifically binds to the carboxyl-terminal regulatory domain of Akt, thereby negatively regulating Akt activity to prevent tumor genesis [16]. Since KA induces excitotoxic brain injury, we investigated the regulation of CTMP on Akt activity in reactive gliosis. The injury-induced changes of CTMP and its regulation in astrogliosis may aid the mechanism of neuronal protection and adaptation in response to cell damage.

**MATERIALS AND METHODS**

**Experimental animals and lesions**

Male imprinting control region (ICR) mice (23–25 g body weight) were obtained from Samtako (Korea). The mice were housed in a controlled environment and provided with *ad libitum* food and water. All animal-related procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University (CNU-00151). We used a previously described procedure with minor modifications [17, 18]. Briefly, 5 gm/ml of KA (Sigma, MO, USA) was prepared in sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4). KA was injected at right lateral cerebral ventricle (anteroposterior (AP); -0.4 mm; mediolateral (ML), 1 mm; dorsoventral (DV); -2.3 mm relative to bregma) using a 50-µl Hamilton microsyringe fitted with a 26 G needle inserted to a depth of 2.4 mm (0.1 µg/5 µl in PBS, i.c.v.). Control mice received an equal volume of saline. Mice were allocated into KA-injected animals (n=6–8 per group) and saline-injected control animals (n=6–8/group). After the injection, the needle remained in place for an additional 5 min before being slowly retracted. The mice were monitored for 6 hours after KA treatment to determine the onset time of seizures. The severity of the seizures was classified into 5 stages: stage 1, facial movements; stage 2, head nodding and myoclonic twitching; stage 3, forelimb clonus with lordotic posture; stage 4, forelimb clonus with reared posture; and stage 5, tonic-clonic seizures without postural control [14, 19]. The mice showing at least stage 3 seizures were considered positive for seizure onset. At 1, 3, and 7 days after KA or saline injection, mice were anesthetized using our established protocol [17, 18]. Frozen coronal sections (40 µl thick) were obtained using a Leica cryostat (CM3050, Deerfield, IL, USA).

**Production of phospho-specific antibodies against CTMP**

Polyclonal antisera (p-CTMP) that recognize specific phosphorylation sites were raised against PRPELRSFSEEVILKDC (Ser-37; 29–46 aa), where the phosphorylated amino acids are underlined. Furthermore, antisera were prepared by simultaneously immunizing with the phosphoserine peptide. The phospho-peptides were coupled with Keyhole-Limpet hemocyanin and injected into rabbits. After purification by Protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) chromatography, the resultant antibodies were affinity-purified using antigenic peptides coupled to Affi-Gel 10 or 15 (Bio-Rad, Hercules, CA). All procedures were performed at 4°C.

**Immunohistochemistry and double Immunofluorescence**

Parallel free-floating sections were blocked with endogeneous peroxidase with 1% H2O2 in PBS, followed by treatment with blocking buffer (0.3% Triton X-100 and 1% fetal bovine serum [FBS] in PBS for 30 min). Samples were then incubated with primary anti-CTMP (1:100, Santa Cruz Biotechnology, CA. #sc-390353,) overnight. Immunohistochemical staining of the tissue sections was performed using the avidin–biotin peroxidase complex (ABC) method described previously [20, 21]. Simultaneous staining of antigens for the double immunofluorescent experiments were performed by using Cy3-conjugated anti-rabbit IgG (Amersham, UK) for pCTMP and p-CREB (Ser133, Upstate Biotechnology, Danvers, MA, #sc-28342) and Cy2-conjugated anti-mouse IgG (Amersham Pharmacia Biotech) for glial fibrillary acidic protein (GFAP, 1:1000, #AM020, Biogenex, San Ramon, CA). Nucleus staining was performed with
Astrocytic Expression of CTMP Following an Excitotoxic Lesion in the Mouse Hippocampus

DAPI. Axiophot microscope (Carl Zeiss, Germany) was used for the analysis of double-stained sections.

**Primary astrocyte culture**

Rat primary cerebral astrocytes were purified from neonatal rats according to standard procedures [20]. Sprague-Dawley rat pups (postnatal day 1, P1) (Samtako, Korea) were decapitated in an ice-chilled dish, and the brains were harvested. After removal of the meninges, the cerebral cortex was dissected and dissociated in dissection media. After centrifugation, the cells were seeded into poly-L-lysine-coated 175 flasks and maintained in Minimal Essential Medium (MEM)-based growth media. The background value was subtracted and a densitometer quantitated the relative intensity (area density) of the bands of interest. Data are presented as percentage change from a blank band. The results were calculated as the ratio of the bands of interest. Light-1 films (Kodak, USA).

Supersignal ECL kit (Pierce Chemical, Rockford, IL) and Biomax for immunolabeled proteins by chemiluminescence using a peroxidase antibody staining. Samples were washed 3 times then detected (1:2000 dilution) (Vector, location?) was used for secondary antibodies (Sigma, USA) in blocking solution. Membranes were washed in TBST (3 times, 10 min each). Peroxidase (1:2000 dilution) (Vector, location?) was used for secondary antibody staining. Samples were washed 3 times then detected for immunolabeled proteins by chemiluminescence using a Supersignal ECL kit (Pierce Chemical, Rockford, IL) and Biomax Light-1 films (Kodak, USA).

**Statistical analysis**

Western blot was quantified by using ImageJ software (NIH), and a densitometer quantitated the relative intensity (area density) of the bands of interest. The background value was subtracted from a blank band. The results were calculated as the ratio change from the corresponding control bands. Data are presented as means±S.D. of the three independent experiments. Students' t test (SPSS version 12.0 software, SPSS Inc.) was used for the results analysis where p<0.05 (*) was considered significant, and p<0.01 (**), p<0.001 (***”) were considered highly significant.

**RESULTS**

**Upregulation of CTMP and p-CTMP expression in astrocytes following KA-Induced excitotoxicity**

The intracerebroventricular (i.c.v.) KA injection is a commonly used excitotoxicity model that promotes seizures and selective hippocampal cell death in mice [22, 23]. Previously, we showed a pyramidal cell loss and glial activation in the hippocampus of this ICR mouse model [18, 20]. In those studies, a typical loss of pyramidal neurons in the hippocampal CA3 regions was apparent on day 1 post-injection of KA, and glial activation had increased from day 1 to day 3 and return to normal at day 7. In order to investigate the putative involvement of CTMP in KA-induced hippocampus excitotoxicity, CTMP expression in the ipsilateral hippocampus of KA-treated mice were monitored. In control mice, there was a weak signal for CTMP in parts of the hippocampus, including CA1, CA3 and the DG (Fig. 1A, E and I). However, CTMP was distinctly increased throughout the hippocampus at 3 days after KA treatment (Fig. 1C, G and K). Slides at 3 days revealed the morphology of star-shaped glial-like cells, detected by the anti-CTMP antibody, which is newly appeared in association with brain injuries (Fig. 1K). To further confirm the cell type in which CTMP was found, we employed double staining with the anti-CTMP and anti-GAFP (astrocytic marker) primary antibodies. CTMP was found to be localized to astrocytic nuclei, indicating that CTMP expression increased in the activated astrocytes in the hippocampi of KA-treated mice (data not shown). To investigate the role of phosphorylation of CTMP on Akt signaling pathway, we examined p-CTMP expression in the KA-treated mice hippocampus. The results showed that KA treatment induced an increase in p-CTMP expression in the astrocytes of hippocampus at 1 day after KA treatment (Fig. 2). As shown in Fig. 1 and 2, CTMP and p-CTMP occurred in astrocytes, indicating that CTMP expression and its phosphorylation predominantly occurred in activated astrocytes in the KA-treated mice hippocampus.

**Time dependent expression of p-CTMP, Akt, CTMP and CREB in astrocyte activation**

CTMP is known as a negative regulator of the Akt pathway and increased CTMP expression lead to decreased phosphorylation on both Ser473 and Thr308 residues of Akt [15]. In contrary, CTMP...
Fig. 1. Representative photograph with immunohistochemical staining with anti-CTMP primary antibodies in kainic acid (KA) treated mice. Mice were administrated with KA for 1 (B, F, J), 3 (C, G, K) and 7 days (D, H, L). Brain sections were collected and stained for CTMP antibody. In the control (A, E, I), CTMP was weakly found in the hippocampus. In the KA-injured hippocampus, a strong staining was observed at 1 day post-lesion and became maximal at 3 days and return to normal level at 7 days. Higher magnification of hippocampus showed sequential changes of CTMP-positive cells. Note that CTMP-positive cells exhibited the star-shaped morphology (I–L). (Scale bars=200 μm in A–D, 100 μm in E–H, 20 μm in I–L).

Fig. 2. Double immunofluorescence staining for the identification of p-CTMP-positive cells in the hippocampus following KA treatment. Mice were administrated with KA for 1 (B, F, J), 3 (C, G, K) and 7 days (D, H, L). In the control (A, E, I), p-CTMP was co-localized with the glial fibrillary acidic protein (GFAP) in the CA3 region of the hippocampus. After KA treatment, p-CTMP/GFAP-positive cells were markedly increased at 1 day (arrowheads) and then decreased. Nuclear counterstaining was performed with DAPI (blue). (Scale bar=20 μm).
is released from Akt via phosphorylation of CTMP by a currently unidentified kinase which allows Akt to be phosphorylated by PDK1 and PDK2 at Thr308 and Ser473, respectively [24]. Since both CTMP phosphorylation and CTMP upregulation occurred specifically in astrocytes from KA-treated mice (Fig. 1, 2), the CTMP regulation on Akt activity in astrocyte activation by LPS/IFN-γ treatment was monitored in primary astrocytes. LPS/IFN-γ-treatment of primary astrocytes promoted p-CTMP

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**Fig. 3.** LPS/IFN-γ induced sequential expression of p-CTMP, p-Akt, CTMP and p-CREB in primary astrocytes. (A) Serum-starved astrocytes were treated with LPS/IFN-γ for the indicated time period, and western blot analysis was performed using anti-p-CTMP, anti-p-Akt (T308), anti-CTMP and anti-p-CREB. Data are expressed as optical densities and represent means±SEM of three independent experiments (**p<0.01, ***p<0.001). (B) Mice were administrated with KA for 1 (B-B, F-J), 3 (B-C, G, K) and 7 days (B-D, H, L). In the control (B-A, E, I), few p-CREB were co-localized with GFAP in the CA3 region of the hippocampus. After KA treatment, p-CTMP/GFAP positive cells were markedly increased at 3 day (arrowheads) and then decreased. Nuclear counterstaining was performed with DAPI (blue). (Scale bar=20 μm).
and Akt phosphorylation (T308) at 1 h (Fig. 3A, top panel). In addition, CTMP and p-CREB increased in primary astrocytes at 6 hrs after LPS/IFN-γ-treatment (Fig 3A, lower panel). Double immunofluorescent analysis with p-CREB further confirmed that phosphorylation of CREB increased in astrocytes in the hippocampus after KA treatment (Fig. 3B). Taken together, this data indicates that phosphorylation of CTMP may regulate Akt/CREB activation and CTMP expression in astrocytes.

DISCUSSION

Although Akt activation has been previously studied, the details on Akt downregulation after Akt activation remain unclear. Currently, no specific Akt phosphatases have been identified. However, phosphatase inhibition of cells results in an increase in Akt phosphorylation and activity [25]. Akt can also be inactivated by the CTMP, which binds Akt, prevents its phosphorylation, and blocks downstream signaling [15, 24]. Previously we demonstrated Akt activity and its neuroprotective function in astrocytes by using the same model with this study [14, 26]. The study of Kim et al. [26] showed injury-induced astrocytic changes in the levels of phosphorylation of Akt, -GSK3β, and -CREB after KA treatment. In Kim et al., the phosphorylation of Akt, GSK3β, and CREB in astrocytes showed similar regional specificities. Also, the phosphorylation of GSK3β exhibited a temporal pattern similar to that of CREB, whilst the expression of phospho-Akt proceeded that of GSK3 and CREB [26]. In this study, we found that CTMP was noticeably increased throughout the hippocampus 3 days after KA treatment. Therefore, it is reasonable that upregulation of CTMP at 3 days may be for inhibition of the Akt signaling pathway, which is activated in astrocytes after KA treatment.

On the other hand, we demonstrated that CTMP increased at 3 days, but p-CTMP increased at 1 day in the astrocytes of hippocampus after KA treatment. Akt activation occurs via a dual regulatory mechanism that requires both phosphorylation at Thr308 and Ser473 and translocation to the plasma membrane [24]. The generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the inner leaflet of the plasma membrane, following PI3K activation, recruits Akt by direct interaction with its PH domain. At the membrane, PH-domain-containing serine/threonine kinase, PDK1, phosphorylates Akt on Thr308 [27]. Thr308 phosphorylation is necessary and sufficient for Akt activation. However, additional phosphorylation at Ser473 by PDK2 is required for maximal activation [28]. At the membrane, association with CTMP prevents Akt from becoming phosphorylated and fully active. Phosphorylation of CTMP by a currently unidentified kinase releases CTMP from Akt. Akt is then phosphorylated by PDK1 and PDK2 at Thr308 and Ser473, respectively [24]. Moreover we reported that phosphorylation of PDK1 is required for Akt activation in KA-mediated excitotoxic lesion in the mouse brain [14]. In this study, we found that in control mice, there was signaling for CTMP in parts of the hippocampus, including CA1, CA3 and the DG (Fig. 1). Miyawaki et al reported CTMP is critical to ischemia-induced neuronal death. They also found that global ischemia in intact rats triggered expression and activation of the CTMP in vulnerable hippocampal neurons and that CTMP bound and extinguished Akt activity and was essential to ischemia-induced neuronal death [29]. This discrepancy may be due to the difference of animal model. However, they did not examine the CTMP expression in hippocampus after post-ischemia. In this study, we found that, in control mice, there was a weak signal for CTMP in parts of the hippocampus, but CTMP expression was strongly increased throughout the hippocampus at 3 days after KA treatment. Therefore, it is conceivable that CTMP may play an inhibitory role on Akt activity in normal neurons. Under astrocyte activation (astrogliosis) by KA treatment, enhancement of phosphorylation of CTMP may be for Akt activation, glial cells protection, or adaptive response to damage. To verify p-CTMP and CTMP regulation on Akt activity in astrocytes, we utilized in vitro astrocyte activation status by using LPS/IFN-γ treatment in primary astrocytes. Western blot analysis confirmed that CTMP phosphorylation increased starting from 1 hr with Akt phosphorylation and finally CTMP and p-CREB expression. Time-dependently expression of p-CTMP p-Akt, p-CREB and CTMP indicate that LPS/IFN-γ-induced phosphorylation of CTMP can activate Akt/GSK3β/CREB signaling, whereas lately emerging enhancement of CTMP can inhibit it.

Taken together, we hypothesize that phosphorylation of CTMP may be associated with cell survival. Because the activation of CREB is regulated by complex phosphorylation at Ser-133 contributes many vital processes, including cell survival [30], and enhancing CREB activity in astrocytes have neuroprotective role in acute brain post-injury [31], the importance of regulation of Akt signaling pathways have been emphasized. Therefore we suggest phosphorylation of CTMP as a novel target for neuroprotection and functional repair in KA-induced temporal lobe epilepsy.

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Astrocytic Expression of CTMP Following an Excitotoxic Lesion in the Mouse Hippocampus

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