Inhibition of eNOS/sGC/PKG Pathway Decreases Akt Phosphorylation Induced by Kainic Acid in Mouse Hippocampus

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The serine/threonine kinase Akt has been shown to play a role of multiple cellular signaling pathways and act as a transducer of many functions initiated by growth factor receptors that activate phosphatidylinositol 3-kinase (PI3K). It has been reported that phosphorylated Akt activates eNOS resulting in the production of NO and that NO stimulates soluble guanylate cyclase (sGC), which results in accumulation of cGMP and subsequent activation of the protein kinase G (PKG). It has been also reported that PKG activates PI3K/Akt signaling. Therefore, it is possible that PI3K, Akt, eNOS, sGC, and PKG form a loop to exert enhanced and sustained activation of Akt. However, the existence of this loop in eNOS-expressing cells, such as endothelial cells or astrocytes, has not been reported. Thus, we examined a possibility that Akt phosphorylation might be enhanced via eNOS/sGC/PKG pathway in astrocytes in vivo and in vitro. Phosphorylation of Akt was detected in astrocytes after KA treatment and was maintained up to 72 h in mouse hippocampus. 2 weeks after KA treatment, astrocytic Akt phosphorylation was normalized to control. The inhibition of eNOS, sGC, and PKG significantly decreased Akt and eNOS phosphorylation induced by KA in astrocytes. In contrast, the decreased phosphorylation of Akt and eNOS by eNOS inhibition was significantly reversed with PKG activation. The above findings in mouse hippocampus were also observed in primary astrocytes. These data suggest that Akt/eNOS/sGC/PKG/PI3K pathway may constitute a loop, resulting in enhanced and sustained Akt activation in astrocytes.

Key Words: Akt phosphorylation, Astrocyte, Kainic acid, Nitric oxide

INTRODUCTION

Previous studies indicate that phosphorylated Akt activates endothelial nitric oxide synthase (eNOS), leading to the production of nitric oxide (NO) [1,2]. NO stimulates soluble guanylate cyclase (sGC), which results in accumulation of cGMP and subsequent activation of the protein kinase G (PKG) [3,4]. Elevated eNOS activity is sufficient to activate phosphatidylinositol 3-kinase 3-kinase (PI3K)/Akt signaling via PKG [5], and activation of the PI3K/Akt pathway resulting from PKG activation by NO/cGMP can prevent neuronal cell death [6]. In addition, it has been reported that activation of sGC resulted in increased cyclooxygenase 2 (COX-2) expression via the PKG/Akt/NF-κB pathway [7]. Taken these reports together, there might be a loop that consisted of PI3K, Akt, eNOS, sGC, and PKG, which makes Akt activation sustained. However, the existence of this loop in eNOS-expressing cells, such as endothelial cell or astrocytes, has not been reported. Thus, we examined the possibilities that the Akt phosphorylation might be enhanced via eNOS/sGC/PKG/PI3K pathway in astrocytes in vivo and in vitro. Because Akt phosphorylation can be induced in astrocytes of mouse hippocampus by kainic acid injection, and in primary astrocytes by thrombin treatment (unpublished data), we used a model of KA-induced excitotoxicity and primary astrocytes as in vivo and in vitro model, respectively.

METHODS

Animals and reagents

Male ICR mice weighing 23~25 g were obtained from Folas-International, Ltd. (Seoul, Korea). All of the animal experiments were conducted in accordance with the animal care guidelines of the National Institutes of Health (NIH) and Korean Academy of Medical Sciences (KAMS). Mice were housed five per cage in a room maintained at 22±2°C

ABBREVIATIONS: eNOS, endothelial nitric oxide synthase; KA, Kainic acid; L-NAME, N (G)-nitro-L-arginine methyl ester; NO, Nitric oxide; PI3K, Phosphatidylinositol 3-kinase; PKG, protein kinase G; sGC, Soluble guanylate cyclase.
with an alternating 12/12 h light/dark cycle. Food and water were available ad libitum. KA and L-NAME, aminoguanidine (AG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). KT-5823, wortmannin, and Guanoguanidine (AG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). KT-5823, wortmannin, and Guanoguanidine (AG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). KT-5823, wortmannin, and Guanoguanidine (AG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Drug treatments

More than three mice were used for each group: KA (0.1 μ g/5 μ l), db-cGMP (2.5 μ g/5 μ l) were intracerebroventricularly (i.c.v.) injected according to the procedure established by Laursen and Belknap [8]. Wortmannin (1 mg/kg), L-NAME (50 mg/kg), ODQ (20 mg/kg), and KT-5823 (1 mg/kg) stock solutions were diluted in saline just before intraperitoneal (i.p.) injection, and were administered 1 h prior to KA injection (W+KA, L+KA, O+KA, KT+KA). db-cGMP together with L-NAME was administered 1 h prior to the KA injection (db+L+KA). Vehicle-treated control group (cont) was also prepared. Primary astrocyte, at 70~90% confluence, were treated with thrombin (5 U/ml, Sigma) or SNP (1 mM, Sigma) for 24 h and the following agents were also treated 1 h prior to thrombin or SNP; wortmannin (10 μ M), ODQ (100 μ M), KT-5823 (2 μ M), L-NAME (1 mM), and db-cGMP (500 μ M).

Immunohistochemistry and double immunofluorescence labeling

All mice were transcardially perfused and post-fixed for 4 h in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose, sectioned coronally (40 μ m) on a freezing microtome (MICROM HM 400R, Walldorf, Germany) and collected in cryoprotectant for storage at −20°C. Free floating immunohistochemical staining was performed with anti-rabbit polyclonal Phospho-Akt-Ser473 antibody (1 : 1,000, Cell Signaling Technology, Beverley, MA, USA) as described previously [9]. For double immunofluorescence labeling, sections were co-incubated with anti-mouse monoclonal GFAP (1 : 2,500, Sigma) and one of the following antibodies: anti-rabbit polyclonal p-Akt-Ser473, p-eNOS (1 : 1,000, BD Transduction, Franklin Lakes, NJ, USA). Alexa-488 conjugated goat anti-mouse IgG (green) and Alexa-568 conjugated goat anti-rabbit IgG (red) were used as fluorescent labeled secondary antibodies (Molecular Probes, Eugene, OR, USA).

Analysis of cells exhibiting DNA fragmentation was performed according to the manufacturer’s instructions using terminal deoxynucleotidyl transferase using peroxide-12-UTP nick end labeling (TUNEL) (Roche Molecular Biochemicals, Indianapolis, IN, USA) to label double stranded DNA breaks suggestive of apoptosis as described previously [10].

Immunoblotting

For western blot analysis, the hippocampi were dissected out from KA injected mice, homogenized in 0.25 M Tris-HCl buffer (pH, 6.8) containing 5 mM EGTA, 5 mM EDTA, 1% SDS, and 10% glycerol, with protease inhibitors (2 μ g/μ l aprotinin, 1 μ g/μ l pepstatin, 2 μ g/μ l leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride), and sonicated on ice. The proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were incubated with anti-p-Akt, anti-Akt, anti-p-eNOS, respectively. Detection was performed with HRP-conjugated goat anti-rabbit IgG (1 : 2,000, Jackson Immuno Research, West Grove, PA, USA) or HRP-conjugated goat anti-mouse IgG (1 : 2,000, Jackson Immuno Research). The blots were visualized with enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA) and exposed on ECL film. Gel images in ECL films were scanned and the digital images were quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA). Cells, primary astrocyte, were collected in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride), and sonicated on ice.
mM EGTA, 1 mM EDTA, 0.5% NP-40, with protease inhibitors) and then proceeded as described in western blotting above.

**Primary astrocyte culture**

Primary astrocyte cultures were derived from 1 to 3 day postnatal SD rat (Daeahn Biolink, Seoul, Korea). Briefly, cerebral cortices were dissected out. After removal of the meninges and blood vessels, the cerebral cortices were collected and minced with scalpel. A single-cell suspension was obtained by mechanical dissociation. The dissociated cells were seeded into poly-D-lysine-coated 170 cm² T-flask at a density of 1×10⁶ cm⁻² and maintained at 37°C, 5% CO₂ and 95% air in Dulbecco’s modified Eagle’s medium/nutrient F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen). Medium was changed after 24 h and then every third day thereafter. When cells reached confluence, flasks were gently shaken for 6 h at 180 rpm to remove microglia and oligodendrocytes. After shaking, astrocytes were detached with 0.125% trypsin-EDTA (Invitrogen), and subcultured at 5×10⁵ cells/60 mm flask. Using this method, we confirmed that these cultures contain over 95% astrocytes, as determined by immunostaining for glial fibrillary acidic protein.

**Statistical analysis**

Statistical analysis was performed by Kruskal-Wallis one-way ANOVA. Values were considered significantly different when p value was <0.05. The results are expressed as means±SD from at least three independent experiments.

**RESULTS**

**Akt and eNOS activation by KA treatment in mouse hippocampus**

To determine whether Akt and eNOS phosphorylation occurs after KA treatment, we performed immunohistochemistry and immunoblotting after KA treatment. Phosphorylation of Akt was detected in astrocytes 6 h after KA treatment and was maintained up to 72 h (Fig. 1A, 1B). Two weeks after KA treatment, astrocytic Akt phosphorylation was normalized to control. Immunoblotings also showed the phosphorylation of Akt and eNOS, a downstream target, were increased in a time-dependent manner up to 72 h after KA treatment (Fig. 1B) and then decreased to control level. As eNOS immunoreactivity appeared to co-localize with that of GFAP in the hippocampus (Fig. 1C), and the pattern of astrocytic eNOS phosphorylation was in accordance with that of astrocytic Akt phosphorylation (Fig. 1B), it is believed that the observed change of Akt phosphorylation by KA occurs in astrocytes.

**Effects of L-NAME on KA-induced Akt and eNOS phosphorylation**

For the elucidation of the relationship between astrocytic Akt phosphorylation and eNOS inhibition by L-NAME, we
examined whether Akt and eNOS phosphorylation in astrocytes could be regulated by L-NAME, non-selective NOS inhibitor. Phosphorylation levels of Akt and eNOS were increased at 24 h in KA-treated groups. However, L-NAME administration prior to KA treatment significantly attenuated the KA-induced phosphorylation of Akt and eNOS (Fig. 2A, L+KA).

To confirm the astrocytic Akt phosphorylation after L-NAME treatment, double immunofluorescence staining was performed on free-floating brain sections using antibodies to p-Akt and GFAP. When L-NAME and KA were cotreated, p-Akt immunoreactivity was found to be co-localized mainly with the GFAP in the hippocampus (Fig. 3, L+KA).

**Role of sGC on KA-induced neuronal cell death and Akt and eNOS phosphorylation**

Given the fact that NO stimulates the formation of cGMP via sGC, it is possible that NO produced from eNOS can regulate the level of Akt phosphorylation and neuronal cell death via sGC. When a specific sGC inhibitor, ODQ, was administered prior to KA treatment, the levels of KA-induced phosphorylation of Akt and eNOS were significantly attenuated (Fig. 2B). When ODQ and KA were cotreated, p-Akt immunoreactivity was decreased and found to be co-localized mainly with the GFAP in the hippocampus (Fig. 3, O+KA).

In order to elucidate the effects of the administration of ODQ prior to the KA injection on the KA-induced neuronal cell death, the representative and quantitative analysis of neuronal cell death was determined with cresyl violet and TUNEL staining (Fig. 4, O+KA). TUNEL-positive neurons in the CA3 region were observed more abundantly in mice treated with ODQ prior to KA injection than in KA injection only (Fig. 4B). These results suggest that NO from eNOS might regulate astrocytic Akt phosphorylation via sGC and subsequent inhibition of Akt phosphorylation can aggravate CA3 neuronal death in the hippocampus by KA injection as in L-NAME treatment.

**Regulation of Akt phosphorylation via NO/cGMP-dependent pathway**

sGC, activated by NO, catalyzes the conversion of GTP to the second messenger molecule, cGMP, which activates PKG [5]. We determined whether cGMP-dependent pathway is involved in NO-induced Akt phosphorylation. For the regulation of PKG activity, KT-5823 or db-cGMP were used as a PKG inhibitor or activator, respectively. KT-5823 administration prior to KA treatment significantly attenuated the levels of KA-induced phosphorylation of Akt and eNOS (Fig. 2C). In contrast, db-cGMP treatment per se significantly increased Akt phosphorylation levels. Furthermore, db-cGMP reversed the decreased Akt and eNOS phosphorylation by L-NAME administration (Fig. 2A, db+L+KA). The tendency to p-Akt immunoreactivity in the astrocytes was similar to those of blotting results of the KT-5823 and db-cGMP treatment (Fig. 3). These results suggest that NO from eNOS might regulate astrocytic Akt phosphorylation through NO/cGMP/PKG pathway.

In order to elucidate the effect of PKG inhibition on KA-induced neuronal cell death, the representative and quantitative analysis of neuronal cell death was determined with cresyl violet and TUNEL staining (Fig. 4). TUNEL-positive neurons in the CA3 region were observed more abundantly in mice treated with KT-5823 prior to KA injection than KA injection only (Fig. 4B). However, pretreatment with db-cGMP significantly attenuated L-NAME aggravated neuronal cell damage (Fig. 4B). These results suggest that astrocytic PKG activation by sGC may be related to the...
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neuronal cell death.

**Role of wortmannin on KA-induced neuronal cell death and Akt and eNOS phosphorylation**

In addition, the administration of wortmannin, specific PI3K inhibitor, significantly attenuated the levels of KA-induced phosphorylation of Akt and eNOS (Fig. 2D), which suggests that neuronal and astrocytic Akt phosphorylation is dependent on PI3K activity.

To test the effect of the administration of wortmannin prior to the KA injection on neuronal cell death, the representative and quantitative analysis of neuronal cell death were determined with cresyl violet and TUNEL staining (Fig. 4). TUNEL-positive neurons in the CA3 region were observed more abundantly in the wortmannin pretreated mice than in the KA-only-treated mice (Fig. 4B). These above data suggest that neuronal and astrocytic Akt phosphorylation in i.c.v. KA injection is dependent on PI3K activity. Attenuated neuronal Akt phosphorylation by wortmannin could be a factor that aggravates neuronal death, and also attenuated astrocytic Akt phosphorylation could have influence on neuronal death because of decreased astrocytic support for neuron.

**KA-induced astrocytic Akt phosphorylation**

Double immunofluorescence labeling experiments with anti-phospho-Akt/anti-GFAP revealed that KA induced astrocytic Akt phosphorylation through PI3K, NO, cGMP, PKG signaling pathways. Several inhibitors, L-NAME, a non-selective NOS inhibitor, ODQ, a specific sGC inhibitor, KT-5823, a PKG inhibitor, db-cGMP, a PKG activator, wortmannin, a PI3K inhibitor, can regulate the level of astrocytic Akt phosphorylation (Fig. 3). Therefore, it is suggested that astrocytic Akt phosphorylation is dependent on NO/cGMP/PKG/PI3K signaling pathways.

**In situ verification of a loop for Akt phosphorylation in primary astrocytes**

The in vivo data from the present study indicate that NO, produced from astrocytic eNOS, constitutes a loop to achieve enhanced Akt activation in astrocytes after KA-induced neuronal insult in mouse hippocampus. To confirm the proposed pathway exclusively in astrocytes, we blocked pharmacologically each step of Akt/eNOS/sGC/PKG/PI3K pathway in primary astrocytes (Fig. 5). Thrombin was used to induce astrocyte activation leading to Akt phosphorylation in primary astrocytes, because it was the most potent Akt activator in primary astrocytes among tested stimuli in our study (data not shown). Thrombin resulted in increased Akt phosphorylation in primary astrocytes and thrombin-mediated Akt phosphorylation was attenuated with inhibitors, which suppress each step in the proposed loop (Fig. 4A). Furthermore, treatment of SNP resulted in increased phosphorylation of astrocytic Akt and db-cGMP, a PKG activator, also resulted in enhanced Akt activation (Fig. 4B). These results strongly suggest that the loop including Akt/eNOS/sGC/PKG/PI3K is present in astrocytes.
DISCUSSION

Previously, we showed that KA-induced neuronal death was attenuated by aminoguanidine, selective iNOS inhibitor, but aggravated by L-NAME in ICR mouse hippocampus [10]. There was a limitation that administered L-NAME is not selective eNOS inhibitor. But, given the fact that L-NAME is 20 times more selective to eNOS than iNOS [11] and that iNOS expression was not detected in astrocytes in KA-induced excitotoxicity model (unpublished data), it is reasonable to think that the decrease of Akt phosphorylation in astrocyte by L-NAME administration is due to inhibition of eNOS. eNOS can be activated by Akt-dependent phosphorylation and elevated eNOS activity is sufficient to activate PI3K/Akt signaling via PKG and induce cell migration and angiogenesis through NO/cGMP/PKG in endothelial cells [5]. In PC12 cells, NO exerts its anti-apoptotic effect through a cGMP-dependent activation of the PI3K/Akt signaling pathway [6]. However, the effects and mechanism of astrocytic Akt phosphorylation is largely unknown. Therefore, the hypothesis is intriguing that in a sequential mode, NO activates sGC, sGC increases production of cGMP, cGMP activates PKG, PKG activates PI3K, PI3K phosphorylates Akt, and p-Akt activates eNOS, which can potentially form a loop for enhanced Akt activation.

In the present study, we observed that KA significantly increased astrocytic Akt, eNOS phosphorylation and that NO from eNOS regulates astrocytic Akt phosphorylation through NO/cGMP/PKG/PI3K pathway, suggesting that a loop in the astrocytes is activated to obtain enhanced Akt phosphorylation. Phosphorylated Akt has been shown to play a role in multiple cellular responses, such as cell growth, cell survival, and protein synthesis. Although PI3K is a crucial activator for Akt, increasing evidence has indicated that Akt can be regulated in PI3K independent manners in neurons, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and calmodulin kinase (CaMK) cascades [12,13]. However, our results showed that the administration of PI3K specific inhibitor, wortmannin, significantly attenuated astrocytic Akt phosphorylation, indicating that astrocytic Akt phosphorylation might be regulated mainly via PI3K (Fig. 3).

Several transcription factors are known to play a role in the anti-apoptotic effects of the PI3K/Akt pathway. These include phosphorylated CREB [14,15], and activated NF-κB [16]. Previous studies showed that CREB activation is associated with BDNF and GDNF expression [17,18]. Previously, we showed that melatonin induces Akt phosphorylation and the expression of GDNF in astroglial cells [19]. Enhanced expression of GDNF by melatonin was thought to contribute to the protection of neuronal cell death. Thus, these results indicate that NO/cGMP/PKG pathway-dependent astrocytic Akt phosphorylation can be a critical step in CREB-mediated GDNF expression and neuronal survival. Akt also promotes survival by activating anti-apoptotic NF-κB signaling through IκB kinase (IKK) activation [16]. NF-κB functions as an anti-apoptotic factor through its induction of genes that inhibit apoptosis. NF-κB has been shown to induce the expression of the inhibitor of apoptosis (IAP) protein family (c-IAP1, c-IAP2 and x-chromosome-linked IAP). Recent experimental results of evidence suggest that the transcription of transforming growth factor-β (TGF-β) is activated by NF-κB [20]. TGF-β was shown to induce neuroprotection in vitro and in vivo [21,22]. Viven et al. [23] reported that neuroprotective activity of TGF-β1 against NMDA neurotoxicity is associated with the modulation of tissue plasminogen activator (tPA)/plasminogen activator inhibitor-1 (PAI-1). PAI-1, produced by astrocytes, is up-regulated by TGF-β1 treatment [24,25]. This events leads to modulation of the NMDA-evoked calcium influx in neurons and is responsible for the neuroprotective effects of TGF-β1 against NMDA injury. Also, it was shown that bacterial lipopolysaccharide (LPS)-induced ischemic tolerance in the brain is mediated via TGF-β1 [21,22]. TGF-β can destabilize iNOS mRNA, retard the synthesis of iNOS protein, and accelerate its degradation [26,27]. Also, Pyo et al. [28] reported that wortmannin enhances LPS-induced iNOS expression in microglia in the presence of astrocyte.

In the present study, we have demonstrated that astrocytic Akt phosphorylation might be enhanced through eNOS/sGC/PKG/PI3K pathway, which increased activation of astrocytic Akt may result in the upregulation of pro-survival transcription factor and neuroprotective factor expression. Therefore, astrocytic Akt phosphorylation may play a significant role in pro-survival neuron-glia cross-talks.
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REFERENCES