Arch Pharm Res Vol 34, No 6, 1023-1034, 2011
DOI 10.1007/s12272-011-0620-3

Archives of Pharmacal Research
www.springer.com/12272

Major Role of the PI3K/Akt Pathway in Ischemic Tolerance Induced by Sublethal Oxygen-Glucose Deprivation in Cortical Neurons *In Vitro*

Mohammad Iqbal Hossain Bhuiyan[1,2], Seo Yun Jung[3], Hyoung Ja Kim[1], Yong Sup Lee[3], and Changbae Jin[1]

[1]Integrated Omics Center, Life/Health Division, Korea Institute of Science and Technology (KIST), P.O. Box 131, Cheongryang, Seoul 130-650, Korea, [2]Department of Biomolecular Science, University of Science and Technology (UST), Daejon 305-333, Korea, and [3]Department of Pharmaceutical Sciences, College of Pharmacy and Department of Life and Nanopharmaceutical Science, Kyung Hee University, Seoul 130-701, Korea

(Received September 30, 2010/Revised January 30, 2011/Accepted February 24, 2011)

Ischemic preconditioning can provide protection to neurons from subsequent lethal ischemia. The molecular mechanisms of neuronal ischemic tolerance, however, are still not well-known. The present study, therefore, examined the role of MAPK and PI3K/Akt pathways in ischemic tolerance induced by preconditioning with sublethal oxygen-glucose deprivation (OGD) in cultured rat cortical neurons. Ischemic tolerance was simulated by preconditioning of the neurons with sublethal 1-h OGD imposed 12 h before lethal 3-h OGD. The time-course studies of relative phosphorylation and expression levels of ERK1/2, JNK and p38 MAPK showed lack of their involvement in ischemic tolerance. However, there were significant increases in Akt phosphorylation levels during the reperfusion period following preconditioned lethal OGD. In addition, Bcl-2 associated death promoter (Bad) and GSK-3β were also found to be inactivated during that reperfusion period. Finally, treatment with an inhibitor of PI3K, wortmannin, applied from 15 min before and during lethal OGD abolished not only the preconditioning-induced neuroprotection but also the Akt activation. Concomitant with blockade of the Akt activation, PI3K inhibition also resulted in activation of Bad and GSK-3β. The results suggest that ischemic tolerance induced by sublethal OGD preconditioning is primarily mediated through activation of the PI3K/Akt pathway, but not the MAPK pathway, in rat cortical neurons.

Key words: Ischemic tolerance, Preconditioning, PI3K/Akt, MAPK, Oxygen-glucose deprivation, Cortical neuron

Selected by Editors

INTRODUCTION

Sublethal ischemic insult(s), known as ischemic preconditioning, can induce tolerance against subsequent lethal ischemic insult. Although the preconditioning phenomenon in relation to ischemia was first described in 1964, it drew attention in the late 1980s (Dahl and Balfour, 1964; Kapinya, 2005). Since then this phenomenon has been confirmed in animal models of global and focal ischemia, brain slice preparations and primary cultured neurons, and has also been observed in human beings in the form of short episodes of ischemia without infarction (Gidday, 2006). Although few previous reports have linked neuroprotective event of preconditioning with transcriptional and/or posttranslational modulation of different signaling molecules, the role of these signaling pathways in induction and transduction of endogenous neuroprotection has yet to be revealed precisely to translate this phenomenon into application (Kapinya, 2005).

The mitogen-activated protein kinase (MAPK) family is thought to play an important role in the cellular adaptation to various stimuli including ischemia. The c-Jun N-terminal kinase (JNK) is activated by many

Correspondence to: Changbae Jin, Integrated Omics Center, Korea Institute of Science and Technology (KIST), P.O. Box 131, Cheongryang, Seoul 130-650, Korea
Tel: 82-2-958-5069, Fax: 82-2-958-5059
E-mail: cbjin@kist.re.kr

stress stimuli and plays a key role in ischemic cell death (Borsello et al., 2003), but the beneficial role of JNK in ischemic tolerance has also been reported (Sato et al., 2000). Although p38 MAPK inhibition has been shown to provide neuroprotection in cerebral ischemia (Sugino et al., 2000), its activation has also been positively implicated in ischemic tolerance (Nishimura et al., 2003). The extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway is activated by growth factors and preconditioning treatments to stimulate cell growth and survival (Hetman et al., 1999). The harmful role of ERK1/2 in neuronal cell survival was also reported (Luo et al., 2007). Therefore, the roles of MAPK signaling cascades are complex in neuronal death and survival paradigm, and remain to be elucidated.

Another type of signaling pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is believed to mediate survival signals in a wide range of neuronal cell types. The serine-threonine kinase Akt is activated by a variety of stimuli, including growth factors, traumatic brain injury and ischemia (Ouyang et al., 1999; Noshita et al., 2002). The activated Akt exerts cell survival action by phosphorylating its many cytoplasmic targets such as Bcl-2 associated death promoter (Bad), glycogen synthase kinase-3 (GSK-3), procaspase-9 and cAMP response element-binding protein (Datta et al., 1999).

In our previous study (Bhuiyan et al., 2010), we have shown that ceramide plays an important role in induction of ischemic tolerance in primary cultured cortical neurons. To gain further insight into signaling mechanisms of endogenous neuroprotection, the present study examined the roles of the MAPK and the PI3K/Akt pathways in *in vitro* ischemic tolerance paradigm. In this study, we provide evidence that the PI3K/Akt pathway, but not the MAPK pathway, is primarily involved in ischemic tolerance induced by sublethal oxygen-glucose deprivation (OGD) preconditioning in primary cultured cortical neurons of rats.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium, Neurobasal medium, supplement B27, supplement B27 without antioxidant, glutamine, penicillin/streptomycin, fetal bovine serum and trypsin were purchased from GIBCO BRL. Wortmannin from Cell Signaling Technology and L-glutamic acid from Tocris Bioscience were purchased. All other reagents were obtained from Sigma-Aldrich unless indicated otherwise.

Primary culture of cortical neurons of rats

Primary cultures of cortical neurons were prepared from Sprague-Dawley rats at approximately 17-18 embryonic days according to the method of Brewer (1995) with some modifications. Briefly, neuronal cells dissociated from cerebral cortices of fetal rats were plated on 100-mm dishes (pre-coated with 10 μg/mL poly-L-lysine) at a density of 7.5×10^6 cells/dish in seeding medium consisting of Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 25 μM glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified incubator containing 5% CO_2 and 95% air (normoxia). One day after plating, the seeding medium was removed and replaced with maintenance medium (seeding medium without glutamate) and refreshed twice a week. This serum-free culture condition allowed us to maintain cultures with a very low percentage (< 5%) of glia.

Preconditioning experiments with oxygen-glucose deprivation

To simulate ischemic injury *in vitro*, cultured cortical neurons were exposed to OGD according to the method described by Goldberg and Choi (1993) with little modifications. Briefly, at 6-7 days *in vitro*, culture medium was replaced with glucose-free N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)-buffered saline (5.4 mM KCl, 51.3 mM NaCl, 20 mM HEPES, 1.8 mM $CaCl_2$, 0.01 mM glycine, pH 7.3, bubbled with a gas mixture of 95% N_2 and 5% CO_2 for 30 min) and dishes were immediately transferred to an anaerobic chamber which was previously rinsed with an anaerobic gas mixture (5% CO_2, 5% H_2 and 90% N_2). Cells were maintained at 37°C during appropriate time periods to produce either sublethal (1-h) or lethal (3-h) OGD. At the end of the appropriate periods, OGD was terminated by changing the deoxygenated HEPES buffer to ordinary pre-warmed Neurobasal media containing B27 supplements without antioxidant and returned the cells to normoxic conditions (reperfusion). Ischemic neuronal injury was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24-h simulated reperfusion.

There were five different treatment groups in the OGD preconditioning experiments as shown in Fig. 1:

Control (sham-wash): cultured neuronal cells were incubated with HEPES buffer supplemented with 25 mM glucose for 1 h in normoxia, and then reperfused for 12 h. After 12-h reperfusion, cells were subjected to 3-h sham OGD, and then reperfused for particular periods.

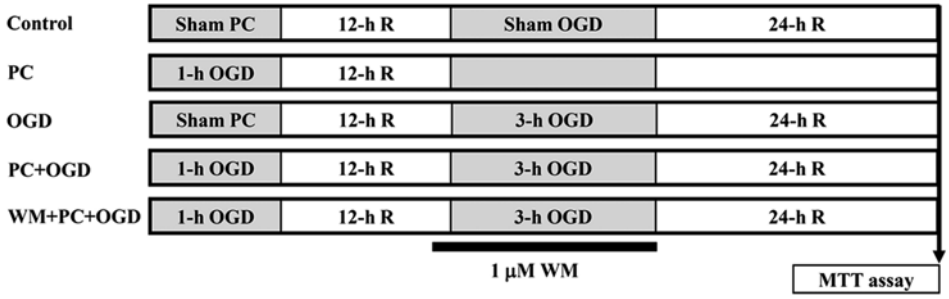

Fig. 1. Experimental protocol of *in vitro* ischemic tolerance in primary cultured cortical neurons of rats. PC, preconditioning; OGD, oxygen-glucose deprivation; R, reperfusion; WM, 1 μM of wortmannin applied from 15 min before and during 3-h OGD

Preconditioning: cells were subjected to 1-h OGD, and then reperfused for 12 h to induce resistance mechanism.

Lethal OGD: cells were subjected to 1-h normoxia in HEPES buffer supplemented with 25 mM glucose, reperfused for 12 h, and then subjected to 3-h OGD and reperfused for particular periods.

Preconditioning + lethal OGD: cells were preconditioned with sublethal 1-h OGD and reperfused for 12 h, and then subjected to lethal 3-h OGD and reperfused for particular periods.

Wortmannin + preconditioning + lethal OGD: it was the preconditioning + lethal OGD group additionally treated with 1 μM of wortmannin, a potent PI3K inhibitor, in 0.05% dimethylsulfoxide (DMSO) applied from 15 min before and during lethal OGD.

Assessment of cell injury

Neuronal cell injury was observed by phase contrast microscopy and quantified by the MTT assay. In brief, fresh medium containing the MTT salt at a final concentration of 0.5 mg/mL was added to cultures and incubated for 2 h at 37°C in a humidified 5% CO_2 incubator with 95% air. After incubation, the medium was aspirated and DMSO was added to dissolve the insoluble purple formazan product into a colored solution which was read at 570 nm using a microplate reader. The absorbance of the formazan formed in the sham-washed control cells was taken as 100% viability.

Western blot analysis

Twenty μg of protein samples were boiled in 1% SDS and 1% β-mercaptoethanol for 5 min, resolved by 10-12% SDS polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene fluoride membrane. The membrane was first blocked with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% skim milk for 1 h at room temperature, and then hybridized with primary antibody by incubating overnight at 4°C. The following primary antibodies purchased from Cell Signaling Technology were used: anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-phospho-p38 (Thr180/Tyr182), anti-phospho-Akt (Ser473), anti-phospho-Bad (Ser136), anti-phospho-GSK-3β (Ser9), anti-ERK1/2, anti-JNK, anti-p38, anti-Akt and anti-Bad antibodies. After incubation with the primary antibody, the membrane was washed with TBST and incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (anti-rabbit antibody, Molecular Probes; anti-mouse antibody, Pierce) for 1 h at room temperature. The resulting immune complex was visualized using an enhanced chemiluminescence Western blotting detection system (ECL, Amersham Biosciences). Protein bands were scanned and digitized with the help of image analysis software (Optimas). Antigens of interest were normalized to the β-actin measured in the same sample.

Statistical analysis

Data were expressed as mean ± S.E.M. All statistical analyses were performed using SPSS and SigmaPlot software (SPSS Inc.). Statistical significance was assessed by the one-way analysis of variance test followed by the Duncan's post-hoc test, and $p < 0.05$ was considered statistically significant. Each experiment was performed at least three times with different batches of cultures.

RESULTS

Neuroprotection induced by sublethal OGD preconditioning

To examine neuroprotective effect induced by ischemic preconditioning, an *in vitro* ischemic tolerance model system was established using primary cultured cortical neurons of rats maintained for 6-8 days in 100-mm culture dishes. Neuronal cell viability was observed

with a phase contrast microscope (Fig. 2A-2D) and quantified by the MTT assay (Fig. 2E) at 24 h of reperfusion following each treatment. The morphological analysis of cortical neurons subjected to lethal 3-h OGD showed extensive neuronal cell damage with most of the neuronal processes degenerated at 24 h of reperfusion following 3-h OGD treatment (Fig. 2C). Neurons preconditioned with 1-h OGD were more resistant to subsequent lethal 3-h OGD, showing most of the neuronal processes intact (Fig. 2D). Quantitative analysis showed that approximately 46% of the neurons survived in the only lethal 3-h OGD-treated group when measured at 24 h of reperfusion following the treatment. On the other hand, preconditioning of the neurons with 1-h OGD produced robust neuroprotection against cell death caused by lethal 3-h OGD imposed 12 h after sublethal OGD, significantly increasing cell viability to 81% compared with that of the only lethal 3-h OGD-treated group (Fig. 2E). No significant loss of cell viability was observed in the l-h OGD treatment group. Morphological observation of cell viability by a phase contrast microscope was well agreed with the quantitative biochemical MTT assay in our *in vitro* ischemic tolerance model.

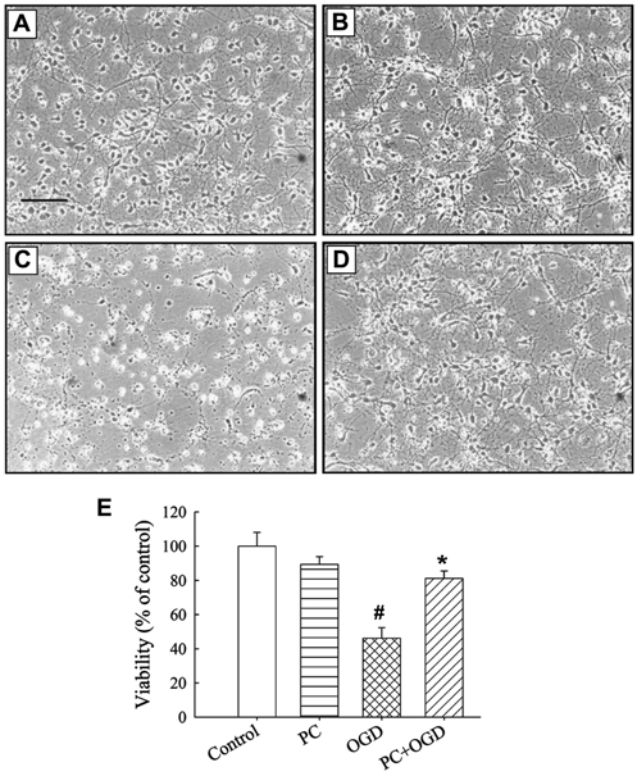

Fig. 2. Morphological photographs and cell viability measurement showing ischemic tolerance induced by sublethal OGD preconditioning (PC) in cultured rat cortical neurons. Phase contrast microscopic photographs of neurons subjected to sham-wash (control, **A**), PC (**B**), OGD (**C**), and PC + OGD (**D**) treatments, were taken following 24-h reperfusion after each treatment. The scale bar is 50 μm. Quantitative cell viability was also measured by the MTT assay following 24-h reperfusion after each treatment (**E**). Values are given as mean ± S.E.M. of at least four independent experiments. $^{\#}p < 0.01$ compared with the control group. $^{*}p < 0.01$ compared with the OGD group. Control, 24 h after sham-wash; PC, 1-h OGD and 24-h reperfusion; OGD, 3-h OGD and 24-h reperfusion; PC + OGD, 1-h OGD and 12-h reperfusion and then 3-h OGD and 24-h reperfusion

No involvement of MAPKs in neuroprotection induced by sublethal OGD preconditioning

To examine whether MAPKs were involved in ischemic tolerance, their phosphorylation levels were measured using a Western blotting method at 12 h of reperfusion following sublethal 1-h OGD, and at 1 h and 24 h of reperfusion following lethal 3-h OGD with or without preconditioning. Our preliminary experiments showed that there were no significant alterations in the phosphorylation and expression levels of proteins of interest in the control and the 1-h OGD-treated group at 1, 12 or 24 h of reperfusion after treatment. Therefore, we chose one relevant time point for each group. In the case of the control group, we chose a time point of 1-h reperfusion after sham OGD to compare with other groups because some proteins of interest were altered at this time point in the OGD and the PC + OGD groups. In the case of the PC group, we chose a time point of 12-h reperfusion after 1-h OGD because preconditioned neurons at this time point showed the highest protection to lethal OGD, although no changes in protein activity or expression levels occurred in this group. Moreover, it was found that it took minimum 1-h reperfusion time after the lethal OGD treatment to observe significant phosphorylation or dephosphorylation of proteins of interest, and those altered levels returned to the basal levels at about 24 h of reperfusion (data not shown). Therefore, those two time points after the lethal OGD treatment were chosen to study the regulation of signaling proteins involved in tolerance induced by ischemic preconditioning. Phosphorylation levels of ERK1/2 were significantly increased at 1 h of reperfusion following lethal 3-h OGD regardless of preconditioning compared with the sham-washed control group and then returned to the basal level at 24 h of reperfusion (Fig. 3A and 3B). In contrast to phospho-ERK1/2 levels, phospho-JNK levels were significantly decreased at 1 h of reperfusion following lethal 3-h OGD regardless of preconditioning compared with the sham-washed control group and then returned to the basal level at 24 h of reperfusion (Fig. 3C and 3D). When phosphorylation levels of p38

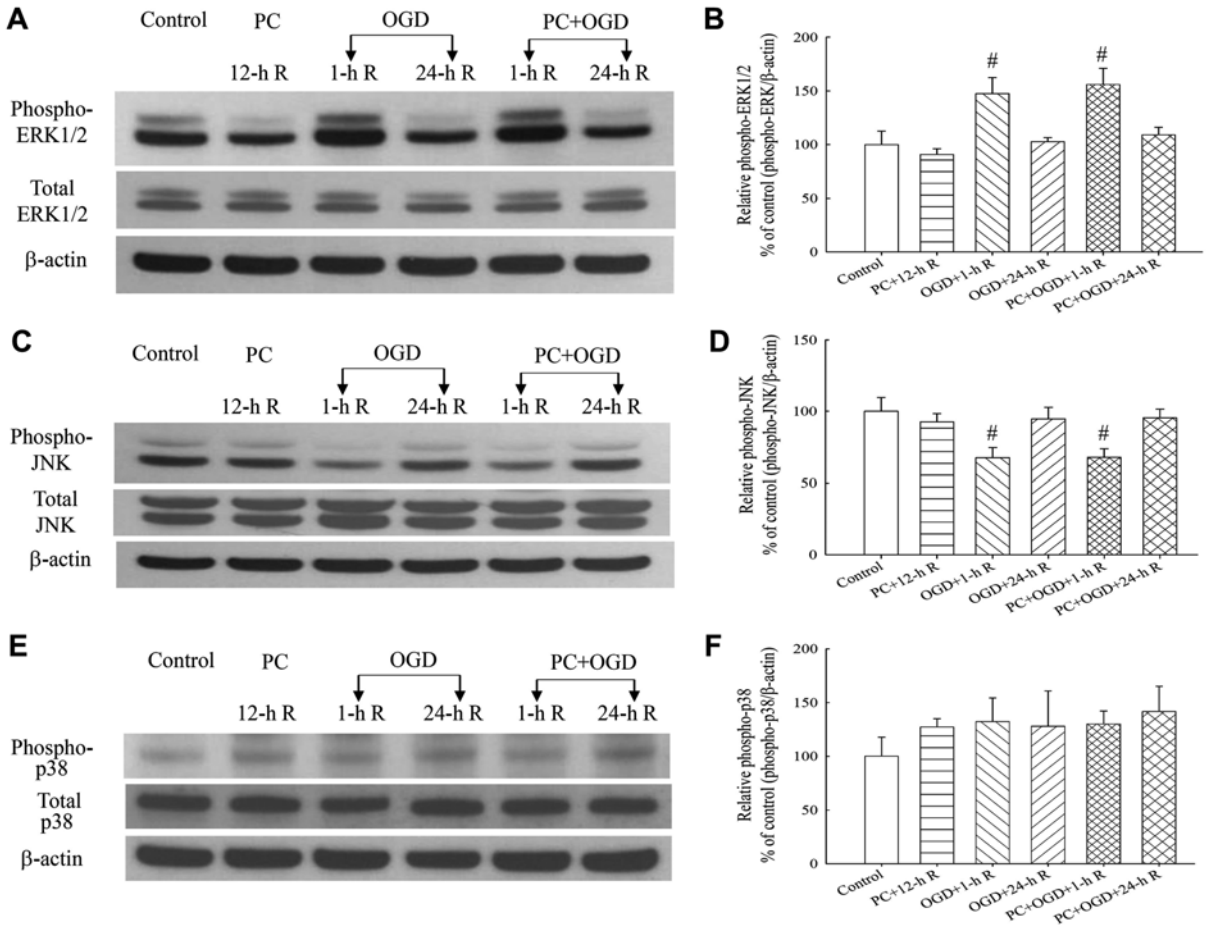

Fig. 3. Temporal profile of phosphorylation levels of ERK1/2 (**A**, **B**), JNK (**C**, **D**) and p38 MAPK (**E**, **F**) during reperfusion period following OGD in cultured rat cortical neurons. Densitometry analyses showed no significant differences in relative phospho-ERK1/2, phospho-JNK or phospho-p38 MAPK levels between OGD and PC + OGD groups. Data are expressed as mean ± S.E.M. of three independent experiments. $^{\#}p < 0.05$ compared with the control group. Control, sham-wash; PC, preconditioning, 1-h OGD; OGD, 3-h OGD; PC + OGD, 1-h OGD and 12-h reperfusion and then 3-h OGD; R, reperfusion

MAPK were measured at 1 h and 24 h of reperfusion following lethal 3-h OGD with or without preconditioning, there were no significant differences between the groups (Fig. 3E and 3F). The finding that preconditioning of the neurons with sublethal 1-h OGD did not produce any significant effects on subsequent lethal 3-h OGD treatment in phosphorylation levels of ERK1/2, JNK or p38 MAPK at the time points tested suggests that the MAPK pathway is not involved in ischemic tolerance induced by preconditioning with sublethal 1-h OGD in the present experimental paradigm.

Increased phosphorylation of Akt induced by sublethal OGD preconditioning

To examine involvement of Akt in ischemic tolerance paradigm, the phosphorylated form of Akt (serine-473) and total Akt protein in the whole cell fraction were immunoblotted (Fig. 4A). Akt was constitutively phosphorylated in the sham-washed control group. Sublethal 1-h OGD treatment did not cause any significant change in phospho-Akt level when measured at 12 h of reperfusion following sublethal 1-h OGD (Fig. 4B). However, the phospho-Akt level in the lethal 3-h OGD group was significantly decreased at 1 h of reperfusion compared with the sham-washed control group and the decreased level did not completely return to the basal level even at 24 h of reperfusion. In contrast, preconditioning of the neurons with sublethal 1-h OGD imposed 12 h before lethal 3-h OGD caused significant increase in the Akt phosphorylation level at 1 h of reperfusion following lethal 3-h OGD, which returned to the basal level at 24 h of reperfusion. Total Akt protein expression levels were similar in all groups. These data indicate that sublethal OGD preconditioning induces phosphorylation of Akt during

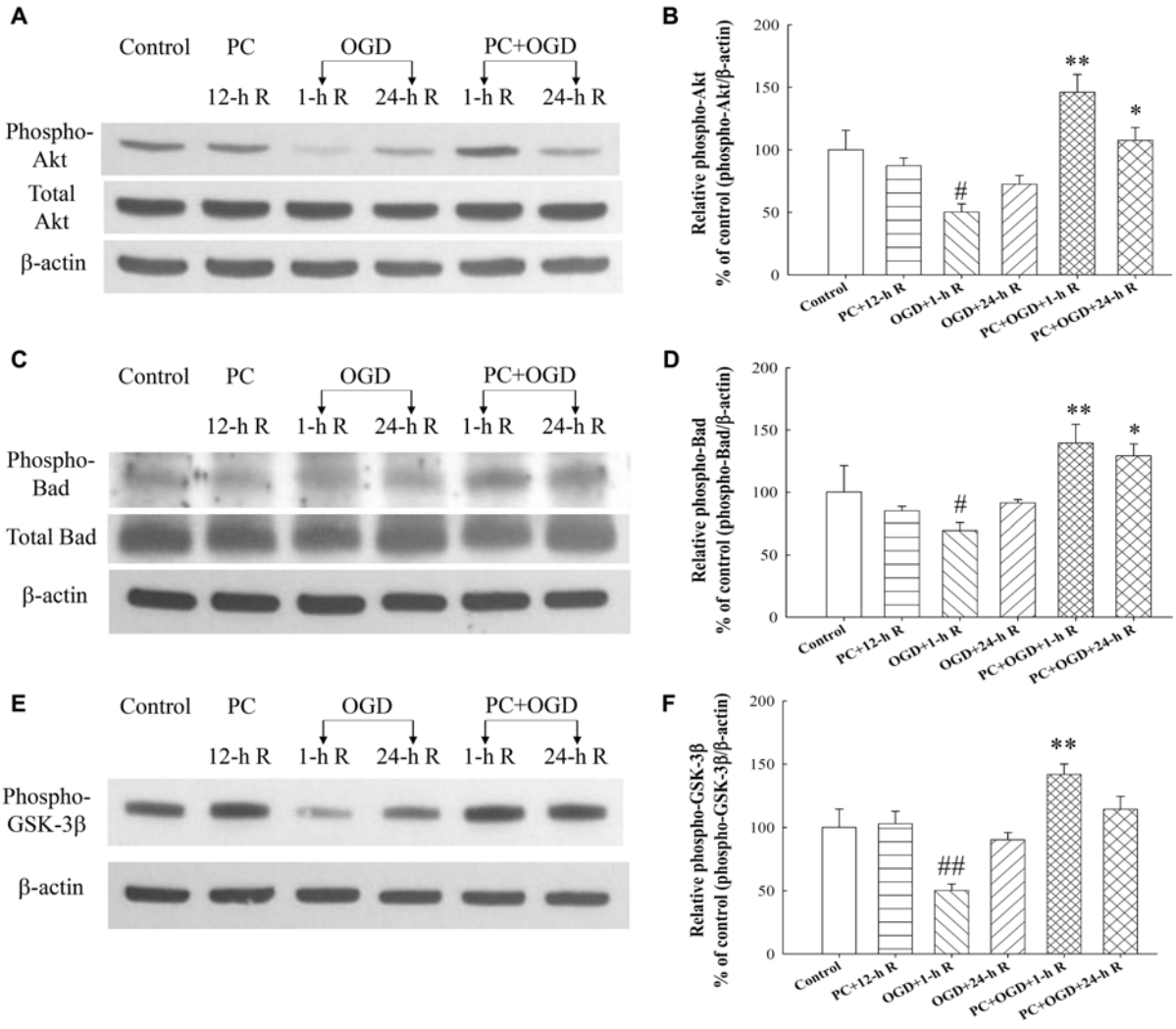

Fig. 4. Temporal profile of phosphorylation levels of Akt (**A**, **B**), Bad (**C**, **D**) and GSK-3β (**E**, **F**) during reperfusion period following OGD in cultured rat cortical neurons. Data are expressed as mean ± S.E.M. of three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the control group. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with the OGD groups at 1-h and 24-h reperfusion times, respectively. Control, sham-wash; PC, preconditioning, 1-h OGD; OGD, 3-h OGD; PC + OGD, 1-h OGD and 12-h reperfusion and then 3-h OGD; R, reperfusion

subsequent lethal OGD/reperfusion stress.

Increased phosphorylation of Akt downstream targets, Bad and GSK-3β, induced by sublethal OGD preconditioning

To examine regulations of Akt downstream targets, Bad and GSK-3β, by the increased levels of phospho-Akt induced by preconditioning, the levels of phospho-Bad and phospho-GSK-3β were measured. Phospho-Bad and phospho-GSK-3β were observed at detectable levels in the sham-washed control group that might be due to the presence of constitutive phospho-Akt in the sham-washed control neurons (Fig. 4C and 4E, respectively). Although the phosphorylated levels of Bad and GSK-3β in the lethal 3-h OGD group was significantly decreased at 1 h of reperfusion compared with the sham-washed control group, preconditioning treatment significantly increased the phosphorylated levels of Bad and GSK-3β at 1 h of reperfusion following lethal 3-h OGD (Fig. 4D and 4F, respectively). The increased levels of phospho-Bad were sustained until 24 h of reperfusion (Fig. 4D). These phosphorylation patterns of Bad and GSK-3β were well paralleled with the pattern of phospho-Akt (Fig. 4). These data imply that increased Akt activity induced by preconditioning inactivates its downstream death-inducing targets through phosphorylation. However, expression levels of total GSK-3β protein were not measured in this

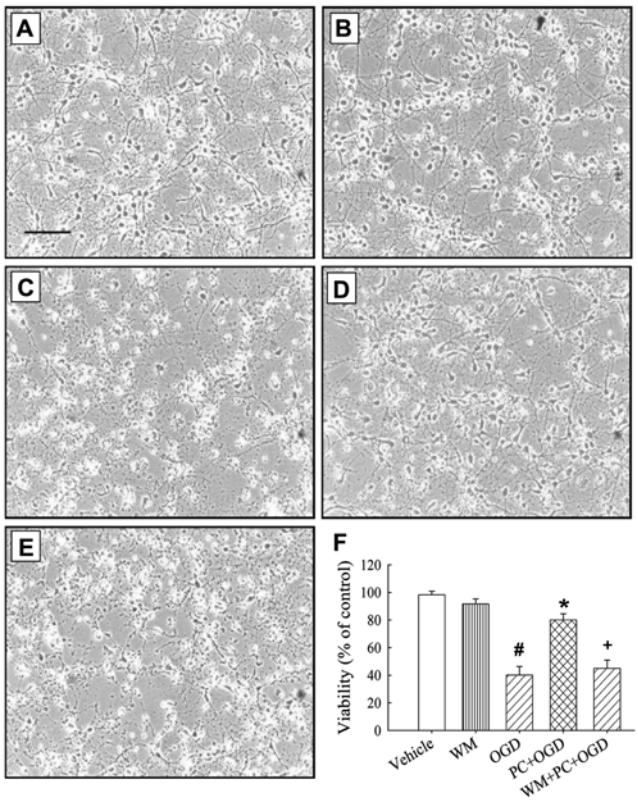

Fig. 5. Morphological photographs and cell viability measurement showing blockade of ischemic tolerance by wortmannin in cultured rat cortical neurons. Phase contrast microscopic photographs of cultured neurons subjected to vehicle (**A**), WM (**B**), OGD (**C**), PC + OGD (**D**) and WM + PC + OGD (**E**) treatments, were taken following 24-h reperfusion after each treatment. The scale bar is 50 μm. Quantitative cell viability was also measured by the MTT assay following 24-h reperfusion after each treatment (**F**). Values are given as mean ± S.E.M. of at least four independent experiments. $^{\#}p < 0.01$ compared with the vehicle-treated control group. $^{*}p < 0.01$ compared with the OGD group. $^{+}p < 0.01$ compared with the PC + OGD group. Vehicle, 0.05% dimethylsulfoxide for 24 h; OGD, 3-h OGD and 24-h reperfusion; PC + OGD, 1-h OGD and 12-h reperfusion and then 3-h OGD and 24-h reperfusion; WM, 1 μM of wortmannin

study since it was reported that this protein levels were not changed under ischemic stress (Endo et al., 2006).

Abolishment of ischemic tolerance induced by sublethal OGD preconditioning by pharmacological blockade of PI3K

To investigate roles of PI3K, an upstream kinase of Akt, in ischemic tolerance, effect of a potent inhibitor of PI3K, wortmannin, on ischemic tolerance induced by sublethal OGD preconditioning was examined. At 1 μM concentration, wortmannin itself did not significantly affect viability of primary cultured cortical neurons (Fig. 5B and 5F). However, treatment of the neurons with wortmannin applied from 15 min before and during lethal OGD completely abolished the neuroprotection produced by sublethal OGD preconditioning against cell death caused by lethal 3-h OGD (Fig. 5E and 5F). In addition, treatment of the neurons with wortmannin during lethal OGD paradigm also completely blocked increased phosphorylation of Akt (Fig. 6A and 6B) and its downstream targets, Bad and GSK-3β (Fig. 6C-6F), which were induced during the reperfusion period following lethal 3-h OGD after preconditioning. However, some variability in Western blotting for total Bad expression levels was observed.

DISCUSSION

Three classical members of MAPK family such as ERK1/2, JNK and p38 MAPK have often been implicated in neuronal cell survival and death mechanisms. In the present study, it was found that at the early stage of reperfusion after lethal OGD, the phosphorylation levels of ERK1/2 were significantly increased compared with the sham-washed control group, whereas those of JNK were decreased. However, preconditioning treatment of the neurons with sublethal OGD did not produce any significant effects on the phosphorylation or protein expression levels of ERK1/2, JNK and p38 MAPK in comparison with the lethal OGD groups. The results suggest that MAPKs are not involved, at least, in ischemic tolerance induced by sublethal OGD preconditioning in primary cultured cortical neurons of rats, which is consistent with several previous reports (Tauskela et al., 1999; Mocanu et al., 2002; Zhou et al., 2007). For example, it was shown that MAPK was not changed following OGD preconditioning and pharmacological inhibition of MAPK by PD98059 did not block preconditioning-induced neuroprotection in cultured rat cortical neurons, suggesting lack of involvement of MAPK in ischemic tolerance (Tauskela et al., 1999). Moreover, it was demonstrated that, although there were some regulations in phosphorylation levels of ERK1/2, JNK and p38 MAPK, these MAPKs were not functionally implicated in the OGD/reperfusion paradigm in rat hippocampal slices (Zhou et al., 2007). In contrast, Gonzalez-Zulueta et al. (2000) demonstrated that 5-min OGD preconditioning induced ERK1/2 phosphorylation, which was required for the development of ischemic tolerance to subsequent 1-h OGD in cultured cortical neurons. Moreover, they showed that treatment with a NMDA receptor antagonist, MK801, during OGD preconditioning completely blocked preconditioning-induced ERK1/2 phosphorylation and also blocked subsequent ischemic tolerance.

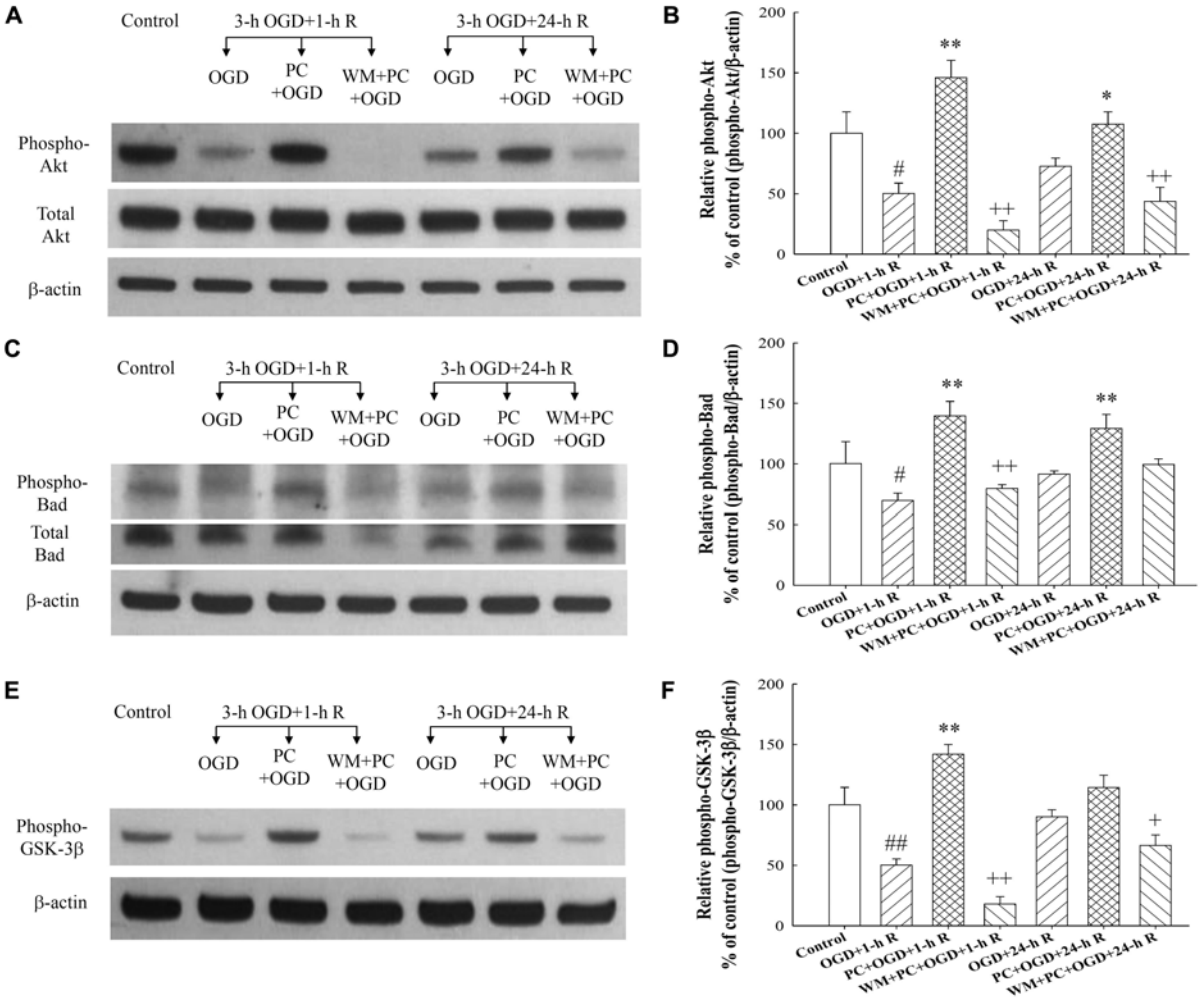

Fig. 6. Blockade of phosphorylation of Akt (**A**, **B**), Bad (**C**, **D**) and GSK-3β (**E**, **F**) induced by sublethal OGD preconditioning (PC) by wortmannin in cultured rat cortical neurons. Data are expressed as mean ± S.E.M. of three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with their respective OGD groups at 1-h and 24-h reperfusion times. $^{+}p < 0.05$ and $^{++}p < 0.01$ compared with their respective PC + OGD groups at 1-h and 24-h reperfusion times. Control, sham-wash; OGD, 3-h OGD; PC + OGD, 1-h OGD and 12-h reperfusion and then 3-h OGD; WM, 1 μM of wortmannin; R, reperfusion

In contrast, it has also been reported that preconditioning treatment with MK801 induces tolerance against subsequent lethal OGD in near-pure primary cultured cortical neurons (Meloni et al., 2002). Although we observed that MK801 provided neuroprotection against lethal 3-h OGD-induced cell death dose-dependently, application of MK801 during OGD preconditioning did not block ischemic tolerance in our study (data not shown). In addition, we could not observe increased activation/phosphorylation of ERK1/2 after sublethal OGD preconditioning in cultured cortical neurons. Instead, increases in ERK1/2 phosphorylation were observed only after lethal OGD regardless of preconditioning, suggesting that strong ischemic insult is required to induce ERK1/2 activation and ERK1/2 activity is not functionally involved in OGD preconditioning-induced neuroprotection in our ischemic preconditioning paradigm. It is noteworthy to mention that the primary cultured cortical neurons used in this study were young (6-8 days *in vitro*) with near-pure neuronal population (< 5% glia), whereas Gonzalez-Zulueta et al. (2000) utilized mature cultured neurons (14 days *in vitro*) with more glial population (10-30%), which might be one of possible reasons for the difference in the signaling mechanisms between these two studies. Interestingly, it was reported that the mechanisms underlying OGD-induced cell death were different in the hippocampal slices from neonatal and adult rats (Zhou and Baudry, 2006). Involvement of JNK was also reported in *in vivo* ischemic and

chemical preconditioning studies where JNK was shown to be negatively regulated by Akt (Zhang et al., 2006). Interestingly, p38 MAPK was reported to play a positive role in ischemic tolerance (Nishimura et al., 2003) or a negative role in hypoxic preconditioning of cortical neurons (Ma et al., 2005). The discrepancies regarding the roles of different MAPKs in ischemia and ischemic tolerance might result from the differences in experimental models (Tauskela et al., 1999; Zhang et al., 2006; Kim et al., 2007), types of insult (Hetman et al., 1999) and age of neurons (Zhou and Baudry, 2006). For example, Hetman et al. (1999) demonstrated that types of cellular injury dictated which kind of neuronal survival pathways, such as ERK1/2 or PI3K/Akt, would be activated to counteract death signals. Although involvement of MAPKs has been reported in some *in vitro* and *in vivo* models of brain ischemia and ischemic tolerance, the differential regulation of MAPKs observed only after lethal OGD in the present study remains to be elucidated.

Lethal ischemia has been implicated in neuronal cell death in a variety of ways such as necrosis, apoptosis, and necroptosis (Wei et al., 2004; Mehta et al., 2007). However, preconditioning has been known to exert robust neuroprotection against every form of cell death during severe ischemic insult (Kapinya, 2005; Gidday, 2006). Therefore, it can be hypothesized that preconditioning acts on both suppression of cell death pathways and activation of cell survival pathways to reduce ischemic cell death. The serine-threonine kinase Akt has been shown to play a vital role in promoting cell survival and inhibiting both apoptotic and non-apoptotic programmed cell death (Datta et al., 1999; Mochizuki et al., 2002). Our results showed that sublethal OGD preconditioning induced activation of Akt through increment of Akt phosphorylation during subsequent lethal OGD/reperfusion stress, which coincided well with cell survival. The results suggest that increased Akt activity induced by preconditioning is related to ischemic tolerance in primary cultured cortical neurons of rats which is in agreement with several *in vivo* ischemic tolerance studies in the brain (Yano et al., 2001; Zhang et al., 2006; Miyawaki et al., 2008). In contrast, no relationship between Akt activity and *in vivo* ischemic tolerance was reported either (Namura et al., 2000). Moreover, it was reported that downregulation of Akt was beneficial in the induction of OGD tolerance in PC12 cells (Hillion et al., 2006). The discrepancies might be due to the differences in experimental models (Tauskela et al., 1999; Hillion et al., 2006), stress stimuli (Hetman et al., 1999), and stress severity (Noshita et al., 2002).

Ample evidence indicates that active Akt exerts cytoprotective effect by counteracting apoptosis, regulating metabolism and activating pro-survival transcription factors (Datta et al., 1999). Therefore, to further elucidate the roles of Akt in OGD tolerance, effects of sublethal OGD preconditioning on two direct downstream targets of Akt, (1) pro-apoptotic Bad and (2) metabolism-regulatory and pro-apoptotic GSK-3β, were examined in the present study. It was found that these death mediators were phosphorylated and inactivated by increased Akt activity induced by OGD preconditioning. Firstly, Bad is a pro-apoptotic member of the Bcl-2 family that can heterodimerise with, and inhibit the survival activity of Bcl-2 and Bcl-X_L proteins (Yang et al., 1995). Phospho-Akt has been shown to promote cell survival via its ability to phosphorylate and inactivate Bad (Datta et al., 1999). Phosphorylation of Bad results in its binding to the adaptor protein 14-3-3 and sequestration into the cytoplasm. Our result showed that phosphorylation of Bad was increased during the reperfusion period in the ischemic tolerant group with increased Akt activity. Although phosphorylation of Bad through the ERK1/2 pathway was also reported (Jin et al., 2002), we found that the ERK1/2 pathway was not involved in the preconditioning-induced neuroprotection. Therefore, it is concluded that active Akt phosphorylates and inactivates Bad to prevent it from binding to Bcl-2 or Bcl-X_L, leading to protection of mitochondrial integrity and neuronal survival in ischemic tolerance paradigm. In fact, it has been reported that the PI3K/Akt signaling pathway is implicated in maintenance of the integrity of the mitochondrial outer membrane (Miyawaki et al., 2008). Secondly, the phosphorylation pattern of GSK-3β, a multifaceted target of Akt, was similar to that of Bad in our OGD tolerant model. GSK-3s are known as pro-apoptotic (Loberg et al., 2002), pro-inflammatory (Guha and Mackman, 2002), and metabolism-regulatory kinases (Parker et al., 1983). There are two highly homologous forms of GSK-3s in the mammals, GSK-3α and GSK-3β. Between these two forms, GSK-3β is particularly abundant in the central nervous system and neuron-specific (Leroy and Brion, 1999). There is growing evidence that GSK-3β is involved in the pathogenesis of neurodegenerative diseases including cerebral ischemia (Sasaki et al., 2001). It has been reported that Akt inactivates GSK-3β through phosphorylation of a serine residue in the amino terminus (Cohen and Frame, 2001). Therefore, effects of sublethal OGD preconditioning on GSK-3β as a crucial downstream target of Akt was also examined. It can be expected that under the conditions where Akt activity is increased, GSK-3β activity is inhibited and

unable to exert its multiple detrimental effects on cell viability through increased phosphorylation. In fact, our findings showed concomitant increases in both Akt activity and GSK-3β phosphorylation in the OGD tolerant model. It was reported that GSK-3β might exert a detrimental apoptotic role through the JNK pathway (Mishra et al., 2007) or the p53 pathway (Turenne and Price, 2001). Since a role of the JNK pathway can be excluded in our model system, p53 may be a putative downstream pathway in harmful action of GSK-3β, which remains to be elucidated.

Finally, to further confirm the role of Akt in ischemic tolerance, effect of a pharmacological inhibitor of its upstream kinase on ischemic tolerance was examined. It is known that Akt is phosphorylated through the PI3K or MAPK pathway (Downward, 1998). Since MAPK was found not to be involved in our ischemic tolerance paradigm, a PI3K inhibitor was chosen. Cultured cortical neurons were treated with a potent inhibitor of PI3K, wortmannin, during lethal OGD paradigm at a concentration of 1 μM known to completely inhibit PI3K (Powis et al., 1994). Treatment with wortmannin applied from 15 min before and during lethal OGD completely blocked not only preconditioning-induced neuroprotection but also increased Akt activation. Concomitant with Akt inactivation, PI3K inhibition also resulted in activation of Akt downstream targets, Bad and GSK-3β. The blockade of preconditioning-induced neuroprotection by wortmannin indicates that neuronal ischemic tolerance induced by sublethal OGD preconditioning is primarily mediated through the PI3K pathway.

Other than anoxia or hypoxia, ischemic tolerance can be induced by diverse preconditioning stimuli such as oxidative stress, hypothermia, heat shock, anesthetics, excitotoxins, cytokines, metabolic inhibitors (Meloni et al., 2002). However, the signaling mechanism responsible for these diverse forms of preconditioning remains mostly a mystery. The present study employed a widely recognized OGD model to simulate ischemia and ischemic tolerance *in vitro* and elucidated the related signaling pathway. It is demonstrated that the PI3K/Akt pathway is involved in ischemic tolerance induced by sublethal OGD preconditioning in primary cultured cortical neurons of rats. It seems that sublethal OGD preconditioning sensitizes the cell survival pathway to subsequent ischemic insult, whose mechanism remains to be elucidated. Recently, we have shown that small increases in endogenous ceramide levels induced by sublethal OGD preconditioning and their presence at the onset of lethal OGD played an important role in neuroprotection against subsequent lethal OGD in primary cultured cortical neurons of rats (Bhuiyan et al., 2010). In addition, cell-permeable ceramide applied exogenously at a non-cytotoxic low concentration was also found to provide neuroprotection against lethal OGD in primary cultured cortical neurons. Therefore, it is likely that there is an interaction between sublethal OGD preconditioning-induced increased ceramide levels and subsequent activation of the PI3K/Akt pathway in ischemic tolerance. In fact, it has been reported that both exogenously added and *de novo* pathway-generated ceramides activate the PI3K/Akt cell survival pathway (Song and Posse de Chaves, 2003).

In conclusion, increased phosphorylation of Akt, Bad and GSK-3β induced by sublethal OGD preconditioning and blockade of preconditioning-induced neuroprotection by a PI3K inhibitor, indicate involvement of the PI3K/Akt-mediated signaling pathways in *in vitro* ischemic tolerance. Although further studies are required to fully clarify the signaling pathways of neuron-specific ischemic tolerance, this study presents multiple lines of evidence to show that the PI3K/Akt pathway, but not the MAPK pathway, plays an important role in mediating neuroprotection induced by sublethal OGD preconditioning against lethal ischemic damage in primary cultured cortical neurons of rats through negative regulation of death promoting Bad and GSK-3β. Understanding the molecular mechanisms of ischemic tolerance may provide new insight into novel therapeutic strategies to limit neurodegeneration in ischemic stroke, traumatic brain injury or other neurodegenerative diseases.

ACKNOWLEDGEMENTS

This research was supported by a grant (PF06216-00) from Plant Diversity Research Center of 21st Century Frontier Research program funded by Ministry of Science and Technology of Korean Government.

REFERENCES

Bhuiyan, M. I., Islam, M. N., Jung, S. Y., Yoo, H. H., Lee, Y. S., and Jin, C., Involvement of ceramide in ischemic tolerance induced by preconditioning with sublethal oxygen-glucose deprivation in primary cultured cortical neurons of rats. *Biol. Pharm. Bull.*, 33, 11-17 (2010).

Borsello, T., Clarke, P. G. H., Hirt, L., Vercelli, A., Repici, M., Schorderet, D. F., Bogousslavsky, J., and Bonny, C., A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat. Med.*, 9, 1180-1186 (2003).

Brewer, G. J., Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and

dentate gyrus. *J. Neurosci. Res.*, 42, 674-683 (1995).
Cohen, P. and Frame, S., The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.*, 2, 769-776 (2001).
Dahl, N. A. and Balfour, W. M., Prolonged anoxic survival due to anoxia pre-exposure: brain ATP, lactate, and pyruvate. *Am. J. Physiol.*, 207, 452-456 (1964).
Datta, S. R., Brunet, A., and Greenberg, M. E., Cellular survival: a play in three Akts. *Genes Dev.*, 13, 2905-2927 (1999).
Downward, J., Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.*, 10, 262-267 (1998).
Endo, H., Nito, C., Kamada, H., Nishi, T., and Chan, P. H., Activation of the Akt/GSK3beta signaling pathway mediates survival of vulnerable hippocampal neurons after transient global cerebral ischemia in rats. *J. Cereb. Blood Flow Metab.*, 26, 1479-1489 (2006).
Gidday, J. M., Cerebral preconditioning and ischaemic tolerance. *Nat. Rev. Neurosci.*, 7, 437-448 (2006).
Goldberg, M. P. and Choi, D. W., Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *J. Neurosci.*, 13, 3510-3524 (1993).
Gonzalez-Zulueta, M., Feldman, A. B., Klesse, L. J., Kalb, R. G., Dillman, J. F., Parada, L. F., Dawson, T. M., and Dawson, V. L., Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc. Natl. Acad. Sci. U. S. A.*, 97, 436-441 (2000).
Guha, M. and Mackman, N., The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.*, 277, 32124-32132 (2002).
Hetman, M., Kanning, K., Cavanaugh, J. E., and Xia, Z., Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J. Biol. Chem.*, 274, 22569-22580 (1999).
Hillion, J. A., Li, Y., Maric, D., Takanohashi, A., Klimanis, D., Barker, J. L., and Hallenbeck, J. M., Involvement of Akt in preconditioning-induced tolerance to ischemia in PC12 cells. *J. Cereb. Blood Flow Metab.*, 26, 1323-1331 (2006).
Jin, K., Mao, X. O., Zhu, Y., and Greenberg, D. A., MEK and ERK protect hypoxic cortical neurons via phosphorylation of Bad. *J. Neurochem.*, 80, 119-125 (2002).
Kapinya, K. J., Ischemic tolerance in the brain. *Acta Physiol. Hung.*, 92, 67-92 (2005).
Kim, E., Raval, A. P., Defazio, R. A., and Perez-Pinzon, M. A., Ischemic preconditioning via epsilon protein kinase C activation requires cyclooxygenase-2 activation *in vitro*. *Neuroscience*, 145, 931-941 (2007).
Leroy, K. and Brion, J. P., Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *J. Chem. Neuroanat.*, 16, 279-293 (1999).
Loberg, R. D., Vesely, E., and Brosius III, F. C., Enhanced glycogen synthase kinase-3β, activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism. *J. Biol. Chem.*, 277, 41667-41673 (2002).
Luo, J., Kintner, D. B., Shull, G. E., and Sun, D., ERK1/2-p90^{RSK}-mediated phosphorylation of Na^+/H^+ exchanger isoform 1. A role in ischemic neuronal death. *J. Biol. Chem.*, 282, 28274-28284 (2007).
Ma, M. C., Qian, H., Ghassemi, F., Zhao, P., and Xia, Y., Oxygen-sensitive delta-opioid receptor-regulated survival and death signals: novel insights into neuronal preconditioning and protection. *J. Biol. Chem.*, 280, 16208-16218 (2005).
Mehta, S. L., Manhas, N., and Raghubir, R., Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res. Rev.*, 54, 34-66 (2007).
Meloni, B. P., Majda, B. T., and Knuckey, N. W., Evaluation of preconditioning treatments to protect near-pure cortical neuronal cultures from *in vitro* ischemia induced acute and delayed neuronal death. *Brain Res.*, 928, 69-75 (2002).
Mishra, R., Barthwal, M. K., Sondarva, G., Rana, B., Wong, L., Chatterjee, M., Woodgett, J. R., and Rana, A., Glycogen synthase kinase-3beta induces neuronal cell death via direct phosphorylation of mixed lineage kinase 3. *J. Biol. Chem.*, 282, 30393-30405 (2007).
Miyawaki, T., Mashiko, T., Ofengeim, D., Flannery, R. J., Noh, K. M., Fujisawa, S., Bonanni, L., Bennett, M. V., Zukin, R. S., and Jonas, E. A., Ischemic preconditioning blocks BAD translocation, Bcl-xL cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A.*, 105, 4892-4897 (2008).
Mocanu, M. M., Bell, R. M., and Yellon, D. M., PI3 kinase and not p42/p44 appears to be implicated in the protection conferred by ischemic preconditioning. *J. Mol. Cell. Cardiol.*, 34, 661-668 (2002).
Mochizuki, T., Asai, A., Saito, N., Tanaka, S., Katagiri, H., Asano, T., Nakane, M., Tamura, A., Kuchino, Y., Kitanaka, C., and Kirino, T., Akt protein kinase inhibits non-apoptotic programmed cell death induced by ceramide. *J. Biol. Chem.*, 277, 2790-2797 (2002).
Namura, S., Nagata, I., Kikuchi, H., Andreucci, M., and Alessandrini, A., Serine-threonine protein kinase Akt does not mediate ischemic tolerance after global ischemia in the gerbil. *J. Cereb. Blood Flow Metab.*, 20, 1301-1305 (2000).
Nishimura, M., Sugino, T., Nozaki, K., Takagi, Y., Hattori, I., Hayashi, J., Hashimoto, N., Moriguchi, T., and Nishida, E., Activation of p38 kinase in the gerbil hippocampus showing ischemic tolerance. *J. Cereb. Blood Flow Metab.*, 23, 1052-1059 (2003).
Noshita, N., Lewén, A., Sugawara, T., and Chan, P. H., Akt phosphorylation and neuronal survival after traumatic brain injury in mice. *Neurobiol. Dis.*, 9, 294-304 (2002).
Ouyang, Y. B., Tan, Y., Comb, M., Liu, C. L., Martone, M. E.,

Siesjö, B. K., and Hu, B. R., Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C, and activation of caspase-like proteases. *J. Cereb. Blood Flow Metab.*, 19, 1126-1135 (1999).

Parker, P. J., Caudwell, F. B., and Cohen, P., Glycogen synthase from rabbit skeletal muscle; effect of insulin on the state of phosphorylation of the seven phosphoserine residues *in vivo*. *Eur. J. Biochem.*, 130, 227-234 (1983).

Powis, G., Bonjouklian, R., Berggren, M. M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., Dodge, J., Grindey, G., and Vlahos, C. J., Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res.*, 54, 2419-2423 (1994).

Sasaki, C., Hayashi, T., Zhang, W. R., Warita, H., Manabe, Y., Sakai, K., and Abe, K., Different expression of glycogen synthase kinase-3beta between young and old rat brains after transient middle cerebral artery occlusion. *Neurol. Res.*, 23, 588-592 (2001).

Sato, M., Cordis, G. A., Maulik, N., and Das, D. K., SAPKs regulation of ischemic preconditioning. *Am. J. Physiol. Heart Circ. Physiol.*, 279, H901-H907 (2000).

Song, M. S. and Posse de Chaves, E. I., Inhibition of rat sympathetic neuron apoptosis by ceramide. Role of p75NTR in ceramide generation. *Neuropharmacology*, 45, 1130-1150 (2003).

Sugino, T., Nozaki, K., Takagi, Y., Hattori, I., Hashimoto, N., Moriguchi, T., and Nishida, E., Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus. *J. Neurosci.*, 20, 4506-4514 (2000).

Tauskela, J. S., chakravarthy, B. R., Murray, C. L., Wang, Y., Comas, T., Hogan, M., Hakim, A., and Morley, P., Evidence from cultured rat cortical neurons of differences in the mechanism of ischemic preconditioning of brain and heart. *Brain Res.*, 827, 143-151 (1999).

Turenne, G. A. and Price, B. D., Glycogen synthase kinase 3beta phosphorylates serine 33 of p53 and activates p53's transcriptional activity. *BMC Cell Biol.*, 2, 12 (2001).

Wei, L., Ying, D. J., Cui, L., Langsdorf, J., and Yu, S. P., Necrosis, apoptosis and hybrid death in the cortex and thalamus after barrel cortex ischemia in rats. *Brain Res.*, 1022, 54-61 (2004).

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J., Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*, 80, 285-291 (1995).

Yano, S., Morioka, M., Fukunaga, K., Kawano, T., Hara, T., Kai, Y., Hamada, K., Miyamoto, E., and Ushio, Y., Activation of Akt/protein kinase B contributes to induction of ischemic tolerance in the CA1 subfield of gerbil hippocampus. *J. Cereb. Blood Flow Metab.*, 21, 351-360 (2001).

Zhang, Q. G., Wang, X. T., Han, D., Yin, X. H., Zhang, G. Y., and Xu, T. L., Akt inhibits MLK3/JNK3 signaling by inactivating Rac1: a protective mechanism against ischemic brain injury. *J. Neurochem.*, 98, 1886-1898 (2006).

Zhou, M. and Baudry, M., Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. *J. Neurosci.*, 26, 2956-2963 (2006).

Zhou, M., Dominguez, R., and Baudry, M., Superoxide dismutase/catalase mimetics but not MAP kinase inhibitors are neuroprotective against oxygen/glucose deprivation-induced neuronal death in hippocampus. *J. Neurochem.*, 103, 2212-2223 (2007).