Ginkgolide B preconditioning protects neurons against ischaemia-induced apoptosis

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Abstract

Ischaemic preconditioning (IP) has been reported to protect the brain against subsequent lethal ischaemia, but it has not been used clinically to prevent ischaemic injury because of safety concerns. The aim of the present study was to see whether Ginkgolide B (GB) is capable of preconditioning as IP to protect neurons against ischaemic injury; if so, which mechanism is involved. Cultured mouse cortical neurons at day 8 were pre-treated with GB (120 μmol/l) for 24 hrs or exposed to short-term ischaemia (1 hr) followed by 24-hr normal culture to induce IP before being treated with severe ischaemia (5 hrs). GB and IP significantly increased cell viability, expression of hypoxia-inducible factor-1 alpha (HIF-1α), erythropoietin (EPO), phosphorylated Bad at serine 136 (^{136}p-Bad) and phosphorylated glycogen synthase kinase- 3β at serine 9 (p-GSK-3β), and decreased the percentage of apoptotic cells and the level of active caspase-3 in severely ischaemic neurons. Moreover, LY294002 that is a specific inhibitor of phosphatidylinositol 3-kinase (PI3K) significantly reduced the enhanced expression of HIF-1α, EPO and ^{136}p-Bad induced by GB and IP. These results suggest that GB, like IP in neurons, is capable of preconditioning against ischaemia-induced apoptosis, the mechanism of which may involve the PI3K signalling pathway.

Keywords: Ginkgolide B ● ischaemic preconditioning ● neuron ● phosphatidylinositol 3-kinase ● apoptosis

Introduction

Ischaemic preconditioning (IP) is a powerful adaptive defence induced by mild ischaemia to increase resistance to future injurious ischaemia [1]. IP has aroused increasing attention in recent years because of its potential therapeutic significance [2–4], yet it has not been employed clinically because of safety concerns [5]. It is therefore necessary to seek a safer preconditioning stimulus that is practical and effective, or a biological agent that is able to mimic preconditioning pharmacologically [4–6]. Recent studies have demonstrated that administration of halogenated anaesthetics and noble gas xenon can precondition the heart and the brain and attenuate damage to these organs [5–7].

The aim of the present study was to see whether Ginkgolide B (GB) is capable of preconditioning as mild ischaemia to protect neurons against ischaemic injury, and to discuss possible mechanisms involved. GB is a main active monomer of ginkgolides, a unique group of diterpenes that are found naturally in the leaves of the Ginkgo biloba tree. Although ginkgolides are known to have neuroprotective properties, the actual mechanisms involved are not clear, for ginkgolides are a complex mixture containing ginkgolide A, B, C, J and various other constituents [8, 9]. We chose GB in this study because it has been evidenced that GB is responsible for the neuroprotective role of ginkgolides [10]. Administration of GB improved stroke index scores and mitochondrial respiration following occlusion of the bilateral carotid arteries in gerbils [8].

Our recent studies [9, 11, 12] showed that ginkgolides had protective effects against hypoxic injury in PC12 cells or neurons and could mimic IP to protect C6 cells against ischaemic injury by up-regulation of hypoxia-inducible factor-1 alpha (HIF-1α), a major regulator of oxygen homeostasis [13], as well as its target

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gene erythropoietin (EPO). The increased content of HIF-1α is a key factor associated with the neuroprotective role in ischaemic/hypoxic preconditioning [13]. However, it is unknown whether GB, as a primary constituent of ginkgolides, has the effect of preconditioning on preventing ischaemic neurons from apoptosis. In this study, we observed the effects of pre-treatment with GB or IP on expression of HIF-1α, EPO as well as caspase-3 in ischaemic neurons and the related signalling pathway, to see whether GB is capable of preconditioning as mild ischaemia to protect neurons against ischaemia-induced apoptosis.

Materials and methods

ICR mice were provided by the Animal House of Nantong University Medical Center (Nantong, JS, China). The Health Department of Nantong Government and the Animal Ethics Committee of Nantong University approved the use of animals for this study.

Neuronal culture

Cultures of pure cortical neurons were obtained from embryonic day 14 to 15 ICR mice as described previously [11]. In brief, the cortical neurons were suspended in complete DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA, USA) containing 2.5 mmol/l glucose and 10% foetal bovine serum (Hyclone, Logan, CA, USA), and plated into poly-L-lysine-coated 6-well or 96-well plates (Corning) at a density of 2.4×10^5 cells/cm^2. Cultures were kept in an incubator at 37°C, 5% CO$_2$ (NAPCO 5400). The medium was replaced by Neurobasal medium with supplemental B27 (Invitrogen) 12 hrs after cells were seeded. Arabinoside was added to the medium on day 3 of culture and remained in the medium for 24 hrs at 10 μmol/l concentration to inhibit non-nerve cell further proliferation [14]. Purity of the cortical neuron cultures was also assessed by staining for the neuron marker neuron-specific enolase. It approached >97% positive cells. The cultures were used for experiments after 8 days *in vitro*.

Experimental design

For ischaemic treatment, the neurons were exposed to a combined condition in which the medium was changed with DMEM without glucose (Invitrogen) and serum-free and 1% O$_2$ in a dedicated incubator (NAPCO 7101FC-1) with 1%O$_2$, 94%N$_2$ and 5%CO$_2$ at 37°C. The neurons were pre-treated with GB (120 μmol/l; Tauto Biotech) in complete DMEM for 24 hrs and then washed for three times to remove GB before being treated with ischaemia for 5 hrs. For IP, neurons were pre-treated with ischaemia for 1 hr followed by 24-hr normal culture before being treated with ischaemia for 5 hrs. To understand whether the effects of GB and IP were associated with activation of the phosphatidylinositol 3-kinase (PI3K) signalling pathway, the neurons were pre-incubated with 50 μmol/l LY294002 (Sigma, St. Louis, MO, USA) for 1 hr and then treated with GB (120 μmol/l) for 24 hrs or ischaemia for 1 hr. The detections of neuronal viability and expression of various proteins were performed immediately after neurons were injured by ischaemia for 5 hrs.

Assessment of cell viability

Cell viability was assessed using a MTT assay or Lactate dehydrogenase (LDH) release as described previously [11]. In MTT assay, the yellow 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyl-monotetrazolium bromide (BDH Chemicals, Poole, England) was reduced to a purple formazan by mitochondrial dehydrogenase in live cells. The results were expressed as a percentage of absorbance measured in control cells. LDH release is a reliable index of cellular injury [11]. The LDH activity in the medium was calculated and converted to percentage of control levels. Morphological changes of neurons were observed using an inverted research microscope (Leica, Wetzlar, Germany) as described previously [11].

Detection of apoptotic nuclei by Hoechst 33342 staining

Neuronal apoptosis was observed by fluorescence microscopy with Hoechst 33342 staining. Rounded cells with condensed or fragmented nuclei were considered apoptosis. The method was described elsewhere [15]. Briefly, coverslips with adherent neurons were collected and washed by PBS after ischaemia, and the neurons were fixed with 4% paraformaldehyde for 1 hr and stained with Hoechst 33342 (5 μmol/l, Sigma) for 1 hr at 4°C. After being mounted with 10% glycerol, the cells were observed under a fluorescent microscope (Leica, Wetzlar, Germany) equipped with a 350 nm excitation laser and a digital camera (Canon, Japan). This experiment was repeated at least twice.

Analysis of apoptosis rate by flow cytometry

Neurons were treated with 0.05% trypsin for 5 min. The cells were detached by shaking in order to bring them into suspension. The cells were washed by centrifugation with cold PBS for three times, and then placed in 75% alcohol at −20°C for 24 hrs to fix sufficiently. The neurons were washed with PBS again to remove alcohol, incubated with 50 mg/l propidium iodide (PI; Sigma) staining solution containing RNase A (15,000 unit/ml) at 4°C for 30 min., and then were sieved through a strainer of 70 μm diameter before cytofluorometric analysis [16]. Flow cytometry was carried out using a FACSCalibur (Becton Dickinson, Franklin Lanes, NJ, USA). The red fluorescence (emission 637 nm) for PI was acquired. A total of 20,000 cells per sample were collected and analysed in CellQuest software (Becton Dickinson).

Western blot analysis

Neurons were harvested in 150 μl lysis buffer containing protease inhibitors [12]. Proteins (60 μg) were separated by a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline, incubated overnight at 4°C with the indicated primary polyclonal rabbit anti-HIF-1α (1:500; Novus Biologicals, Littleton, CO, USA), EPO (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated Bad at serine 136 ([136]p-Bad), Bad, phosphorylated glycogen synthase kinase- 3β at serine 9 (p-GSK-3β), GSK-3β and caspase-3 (1:500; Cell Signaling Technology, Beverly, MA, USA) antibody, and then incubated with the secondary antibody (1:2000; goat anti-rabbit IgG antibody linked to horseradish peroxidase; Pierce Biotechnology, Rockford, IL, USA) for 2 hrs
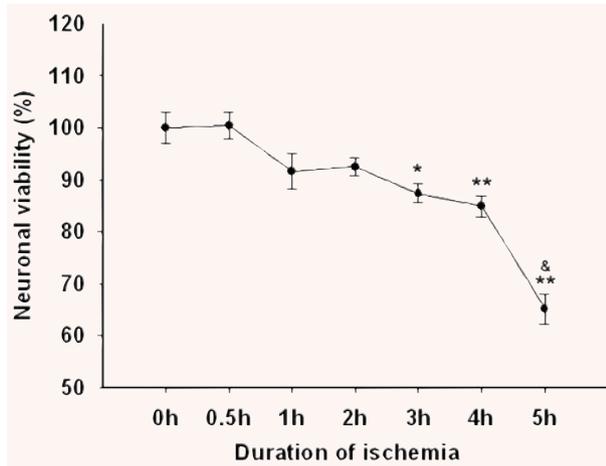
Fig. 1 Effects of ischaemia in different periods on neuronal viability. Neurons were treated with 1%O$_2$ and DMEM without glucose and serium-free for 0, 0.5, 1, 2, 3, 4 and 5 hrs. Neuronal viability was measured by MTT assay. Data were normalized by control as 100% ($n = 18$). *$P < 0.05$ *versus* control (0 hr ischaemia); **$P < 0.01$ *versus* the control; $^{\&}P < 0.05$ *versus* ischaemia for 4 hrs.
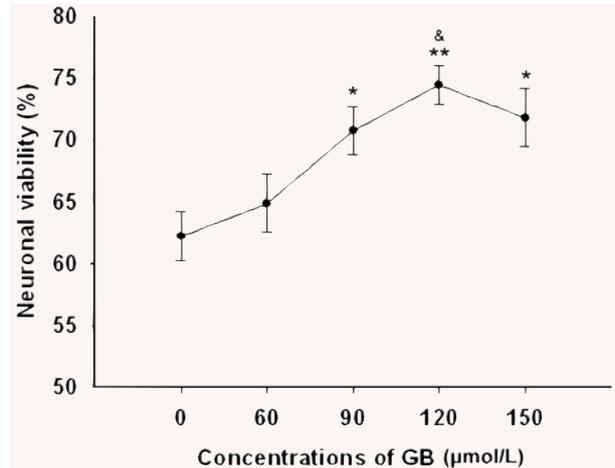
Fig. 2 Effects of different Ginkgolide B concentratins on neuronal viability in ischaemia. Neurons were pre-incubated with 0, 60, 90, 120 and 150 μmol/l of GB for 24 hrs before being treated with ischaemia for 5 hrs. Cell viability was measured by MTT assay. Data were normalized by control as 100% ($n = 18$). *$P < 0.05$ *versus* 0 μmol/l GB; **$P < 0.01$ *versus* 0 μmol/l GB; $^{\&}P < 0.05$ *versus* 60 μmol/l GB.

at room temperature, and finally incubated with ECL Western Blotting Substrate (Pierce Biotechnology) for 5 min. β-actin monoclonal antibody (1:20,000; Sigma) was used as internal protein controls. Immunolabeling was detected using Kodak XAR-5 film for visualization.

Statistical analysis

Data were expressed as mean ± S.D. The difference between means was determined by one-way anova followed by a Student–Newman–Keuls test for multiple comparisons. A probability value of $P < 0.05$ was taken to be statistically significant.

Results

Effects of ischaemia in different periods on cultured neurons

We first measured the viability of neurons in ischaemia for 0, 0.5, 1, 2, 3, 4 and 5 hrs by MTT assay (see 'experimental design'). It was found that cell viability descended with the increase of ischaemic duration, significantly decreased after ischaemia for 4 or 5 hrs, and reached the lowest value (about 65% of control at 0 hr) after ischaemia for 5 hrs (Fig. 1). This result demonstrated that neurons were seriously injured after ischaemia for 5 hrs, but hardly injured after ischaemia for 0.5 or 1 hr. Therefore, ischaemia for 1 hr and ischaemia for 5 hrs were used as mild (ischaemic preconditioning, IP) and severe ischaemia, respectively, in the following experiments.

GB at 120 μmol/l induced the highest increase in viabiliy of ischaemic neurons

We investigated the effects of different GB concentrations on viability of ischaemic neurons. The neurons were pre-incubated with 0, 60, 90, 120 and 150 μmol/l of GB for 24 hrs before being treated with ischaemia for 5 hrs (severe ischaemia). It was found that neuronal viability increased progressively with the GB concentration increasing, reached the peak at 120 μmol/l and then decreased at 150 μmol/l (Fig. 2). This dosage (120 μmol/l) was therefore used in the following experiments.

Both GB and IP protected neurons against ischaemic injury

To find out whether there was any protective effect of IP against ischaemia, the neurons were pre-treated with ischaemia for 0.5 or 1 hr and then exposed to 21% O$_2$ and complete DMEM for 24 hrs before being treated with ischaemia for 5 hrs. Meanwhile, sister neurons were pre-treated with GB for 24 hrs and then exposed to ischaemia for 5 hrs. It was found that cell viability in the ischaemic neurons pre-treated with GB or IP (0.5 or 1 hr) was significantly higher than that in the ischaemic neurons without pre-treatment (All $P < 0.01$ *versus* '0 hr + 5 hrs', Fig. 3B). These data were in agreement with the corresponding morphological changes (Fig. 3A), demonstrating that both GB and IP could significantly protect neurons against ischaemic injury.
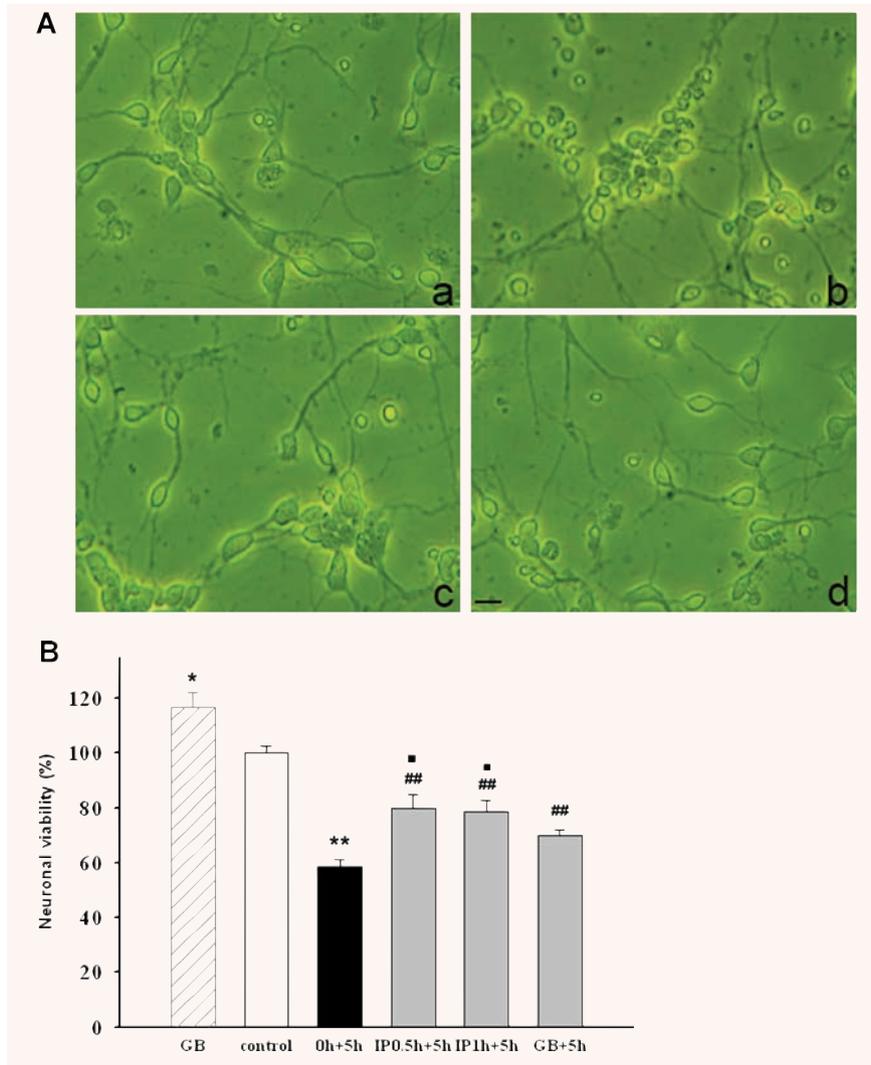
Fig. 3 Effects of Ginkgolide B or ischaemic preconditioning on neuronal viability in ischaemia. Neurons were pre-treated with GB for 24 hrs or IP (0.5 or 1 hr) followed by 24-hr normal culture before exposure to ischaemia for 5 hrs. Control: neurons without any treatment; GB: neurons treated with GB alone for 24 hrs; 0 hr + 5 hrs: neurons in ischaemia for 5 hrs without pre-treatment; IP 0.5 hr + 5 hrs: IP for 0.5 hr + ischaemia for 5 hrs; IP 1 hr + 5 hrs: IP for 1 hr + ischaemia for 5 hrs; GB + 5 hr: GB for 24 hrs + ischaemia for 5 hrs. (**A**) Neuronal morphological changes (a: control; b: 0 hr + 5 hrs; c: IP 1 hr + 5 hrs; d: GB + 5 hrs, bar = 30 μM). (**B**) GB (120 μmol/l) and IP on viability of neurons by MTT assay ($n = 18$). $*P < 0.05$ *versus* control; $**P < 0.01$ *versus* control; $^{\#\#}P < 0.01$ *versus* '0 hr + 5 hrs'; $^{\blacksquare}P > 0.05$ *versus* 'GB + 5 hrs'.

Interestingly, our results also showed that GB was able to raise viability of normal cultured neurons without ischaemic treatment ($P < 0.05$ *versus* 'control'), which was similar to our previous report for ginkgolides [17]. This suggested that GB might have a trophic action on neurons, which also implied the protective effect of GB against severe ischaemia.

Both GB and IP protected neurons against ischaemia-induced apoptosis

To find out whether GB or IP could prevent neurons from apoptosis induced by ischaemia, neurons were pre-treated with GB for 24 hrs or ischaemia for 1 hr followed by 21% O_2 for 24 hrs before being treated with ischaemia for 5 hrs (see 'experimental design'). By Hoechst 33342 staining, nuclear condensation with more bright fluorescence was visualized after ischaemia for 5 hrs under the fluorescent microscope (the bright nuclear condensation pointed by arrows in Fig. 4A and B), whereas the neurons pre-treated with GB or IP had relatively normal nuclei (Fig. 4D).

To further quantitate the apoptosis rate of neurons precisely, neurons that received different treatments were collected and treated with PI staining process before flow cytometric analysis. It was found that the precentage of apoptotic cells in ischaemic neurons pre-treated with GB or IP was significantly lower than that in ischaemic neurons without GB or IP pre-treatment (Fig. 4B). These results demonstrated that pre-treatment with GB and IP both significantly reduced the apoptosis rate in ischaemic neurons.

Then, we investigated the effects of GB or IP pre-treatment on the level of cleaved/active caspase-3, an apoptotic marker [18] in ischaemic neurons. Western blot analysis showed that the level of cleaved caspase-3 in the ischaemic neurons pre-treated with

Fig. 4 Effects of Ginkgolide B or ischaemic preconditioning on ischaemia-induced neuronal apoptosis. Neurons were pre-treated with IP for 1 hr or GB for 24 hrs before exposure to ischaemia for 5 hrs and then stained by Hoechst 33342 or PI. The expression of cleaved caspase-3 was determined by Western blot. Control: neurons without treatment; GB: neurons treated only with GB for 24 hrs; 0 hr + 5 hrs: neurons in ischaemia for 5 hrs without pre-treatment; IP + 5 hrs: IP for 1 hr + ischaemia for 5 hrs; GB + 5 hrs: GB for 24 hrs + ischaemia for 5 hrs. (**A**) Representative photographs of neuronal nucleus by Hoechst 33342 staining (a: control; b: 0 hr + 5 hrs; c: IP 1 hr + 5 hrs; d: GB + 5 hrs, bar = 10 μM). (**B**) Flow-cytometric histograms of PI-stained neurons ($n = 6$). (**C**) A representative experiment of Western blot of cleaved caspase-3; (**D**) Quantification of expression of cleaved caspase-3 protein ($n = 3$). $^*P < 0.05$ *versus* control; $^{**}P < 0.01$ *versus* control; $^{\#\#}P < 0.01$ *versus* '0 hr + 5 hrs'; $^{\blacksquare}P > 0.05$ *versus* 'GB + 5 hrs'.

GB or IP was significantly lower than that in the ischaemic neurons without pre-treatment of GB or IP (Fig. 4C and D), implying that GB or IP could significantly decrease the content of cleaved/active caspase-3 in ischaemic neurons, which might be involved in their anti-apoptotic actions.

Interestingly, we also found that application of GB alone decreased the percentage of apoptosis and level of cleaved caspase-3 in normal cultured neurons without any stimulus (Fig. 4B–D), implying that GB could prevent neurons from apoptosis either in ischaemia or in normal condition.
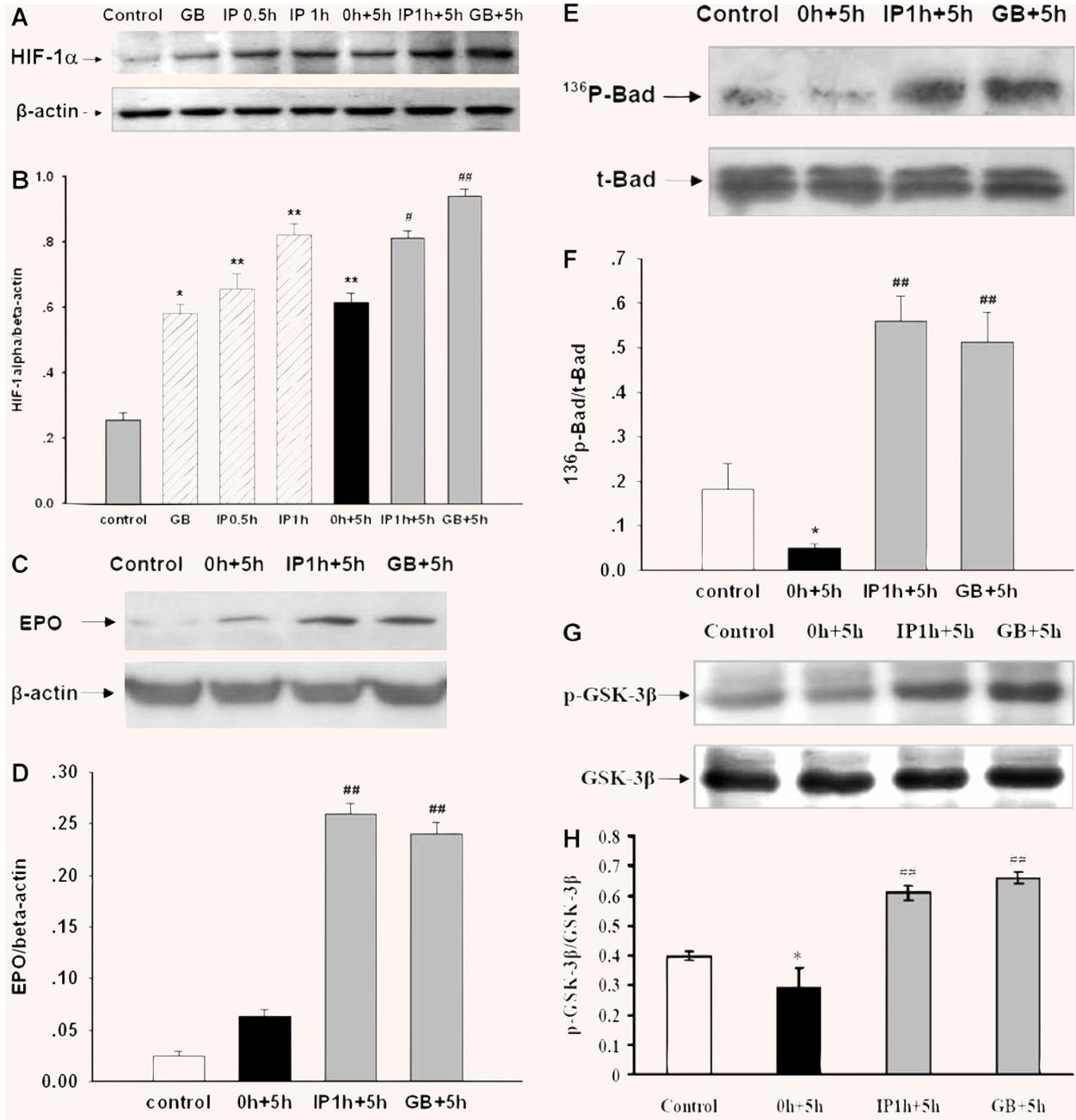
Fig. 5 Effects of Ginkgolide B or ischaemic preconditioning on expression of HIF-1α, EPO, [136]p-Bad and p-GSK-3β in ischaemic neurons. Neurons were pre-treated with or without IP 1 hr or GB for 24 hrs before exposure to ischaemia for 5 hrs and expression of HIF-1α, EPO, [136]p-Bad and p-GSK-3β was then determined by Western blot. (**A, C, E** and **G**) A representative experiment of Western blot of HIF-1α, EPO [136]p-Bad and p-GSK-3β; (**B, D, F** and **H**) Quantification of expression of HIF-1α, EPO, [136]p-Bad and p-GSK-3β protein ($n = 3$). $*P < 0.05$ *versus* control; $**P < 0.01$ *versus* control; [#]$P < 0.05$ *versus* '0 hr + 5 hrs'; [##]$P < 0.01$ *versus* '0 hr + 5 hrs'.
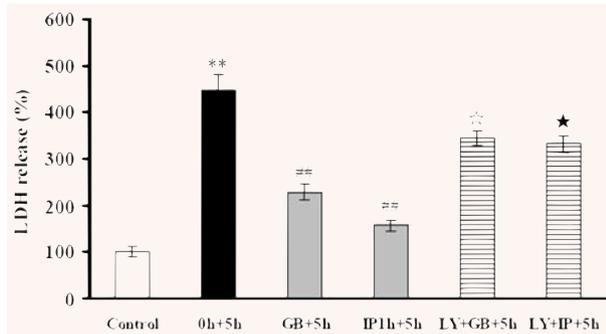
Fig. 6 Effects of PI3K on neuronal viability enhanced by Ginkgolide B or ischaemic preconditioning in ischaemia. Neurons were pre-incubated with or without 50 μmol/l LY294002 for 1 hr before being treated with GB for 24 hrs or ischaemia for 1 hr followed by 24-hr normal culture and then exposed to severe ischaemia. LDH release was normalized by control as 100% ($n = 8$). Control: neurons without any treatment; 0 hr + 5 hrs: neurons in ischaemia for 5 hrs without pre-treatment; GB + 5 hrs: GB for 24 hrs + ischaemia for 5 hrs; IP 1 hr + 5 hrs: IP for 1 hr + ischaemia for 5 hrs; LY + GB + 5 hrs: LY294002 for 1 hr + GB for 24 hrs + ischaemia for 5 hrs; LY + IP + 5 hrs: LY294002 for 1 hr + IP for 1 hr + ischaemia for 1 hr. **$P < 0.01$ *versus* control; ##$P < 0.01$ *versus* '0 hr + 5 hrs'; ☆$P < 0.01$ *versus* 'GB + 5 hrs'; ★$P < 0.05$ *versus* 'IP 1 hr + 5 hrs'.

Both GB and IP induced the expression of HIF-1α, EPO, p-Bad and p-GSK-3β in ischaemic neurons

To understand potential mechanisms involved in GB or IP inhibited neuronal apoptosis induced by ischaemia, we investigated the effects of ischaemia in different periods on expression of HIF-1α in cultured neurons, and examined the effects of pre-treatment with GB or IP on expression of HIF-1α, EPO, [136]p-Bad and p-GSK-3β in severe ischaemic neurons by Western blot. It was found that the increase in level of HIF-1α protein began with ischaemia for 0.5 hr in cultured neurons, the level of HIF-1α protein induced by ischaemia for 1 hr was higher than that induced by ischaemia for 0.5 and 5 hrs (Fig. 5A and B). These results suggested that ischaemia for 1 hr could induce the highest expression of HIF-1α in cultured neurons. In addition, it was found that GB might raise the level of HIF-1α in normal cultured neurons without any other stimulus (Fig. 5A and B). Our results also showed that the levels of HIF-1α, EPO and [136]p-Bad proteins in the ischaemic neurons pre-treated with GB or IP were significantly higher than those in the ischaemic neurons without pre-treatment of GB or IP (Fig. 5). These data demonstrated that GB or IP could significantly induce an increase in the expression of HIF-1α, EPO and [136]p-Bad in ischaemic neurons and that the anti-apoptotic effects induced by GB or IP might be associated with the increased expression of HIF-1α, EPO and [136]p-Bad in neurons.

Meanwhile, it was also found that level of phosphorylation of GSK-3β that is an important kinase of PI3K pathway [18, 19] in the ischaemic neurons pre-treated with GB or IP was significantly higher than that in the ischaemic neurons without pre-treatment of GB or IP, which implied the anti-apoptotic effects of GB or IP could be mediated by PI3K pathway.

Inhibition of phosphatidylinositol 3-kinase decreased the cell viability of ischaemic neurons enhanced by GB or IP

To understand whether PI3K pathway involved in protective effect of GB or IP, we tested the effect of inhibitor of PI3K on cell viability enhanced by GB or IP in ischaemic neurons by LDH release assay. The ischaemic neurons were pre-incubated with or without 50 μmol/l LY294002, a specific inhibitor of PI3K [20], for 1 hr before being treated with GB for 24 hrs or ischaemia for 1 hr followed by normal culture for 24 hrs and then exposed to severe ischaemia. LDH release was normalized by control as 100% ($n = 8$). It is found that LDH release markedly increased after severe ischaemia, the pretreatment of GB or IP significantly decreased the LDH release in ischaemic neurons, which could be reversed by LY294002 (Fig. 6). This further indicated that GB or IP could exert neuroprotective effect through PI3K signalling pathway.

Inhibition of phosphatidylinositol 3-kinase reduced expression of HIF-1α, EPO and p-Bad induced by GB or IP in neurons

To further explore the mechanism of GB or IP in preventing neurons from ischaemia-induced apoptosis, the neurons were pre-incubated with or without 50 μmol/l LY294002 for 1 hr before being treated with GB for 24 hrs or ischaemia for 1 hr. It was found that the expression of EPO and [136]p-Bad in neurons treated with GB for 24 hrs or ischaemia for 1 hr followed by 24-hr normal culture was significantly higher than that in the control without any stimulus, and the expression of HIF-1α was significantly higher than that in the control after neurons were treated with GB for 24 hrs or ischaemia for 1 hr by Western blot. Pre-incubation with LY294002 obviously decreased the expression of HIF-1α, EPO and [136]p-Bad in the neurons treated with GB or IP (Fig. 7). The content of these proteins in the neurons pre-incubated with LY294002 approached the level of control. It also found that LY294002 could not affect the expression of HIF-1α, EPO and [136]p-Bad in the absence of GB or IP (data not shown). These findings indicated that the enhanced expression of HIF-1α, EPO and [136]p-Bad induced by GB and IP could be significantly inhibited by inhibitor of PI3K, which suggested that GB or IP could induce the expression of HIF-1α, EPO and [136]p-Bad in neurons mainly through the PI3K signalling pathway.
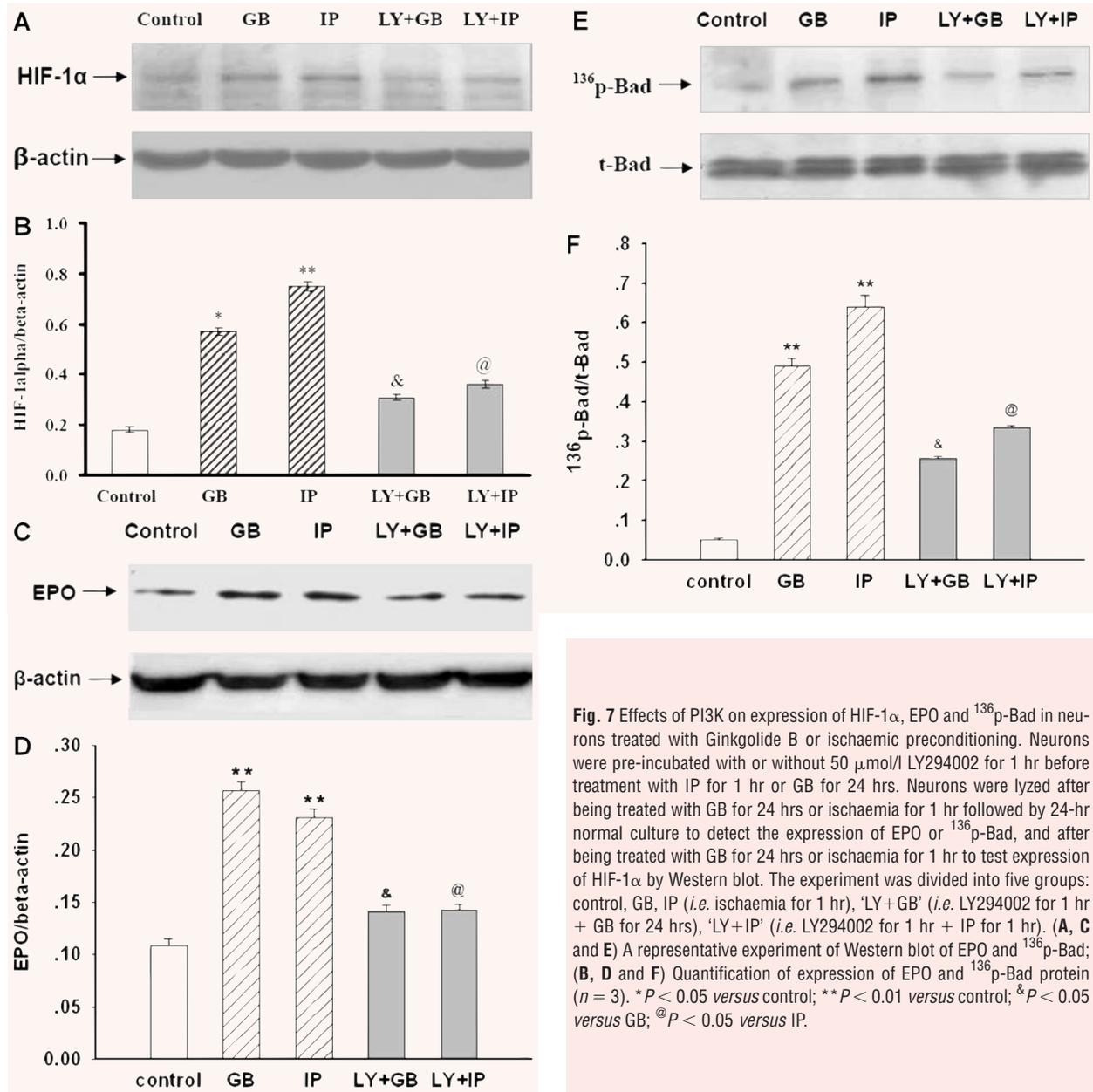
Fig. 7 Effects of PI3K on expression of HIF-1α, EPO and ^{136}p-Bad in neurons treated with Ginkgolide B or ischaemic preconditioning. Neurons were pre-incubated with or without 50 μmol/l LY294002 for 1 hr before treatment with IP for 1 hr or GB for 24 hrs. Neurons were lyzed after being treated with GB for 24 hrs or ischaemia for 1 hr followed by 24-hr normal culture to detect the expression of EPO or ^{136}p-Bad, and after being treated with GB for 24 hrs or ischaemia for 1 hr to test expression of HIF-1α by Western blot. The experiment was divided into five groups: control, GB, IP (*i.e.* ischaemia for 1 hr), 'LY+GB' (*i.e.* LY294002 for 1 hr + GB for 24 hrs), 'LY+IP' (*i.e.* LY294002 for 1 hr + IP for 1 hr). (**A, C** and **E**) A representative experiment of Western blot of EPO and ^{136}p-Bad; (**B, D** and **F**) Quantification of expression of EPO and ^{136}p-Bad protein ($n = 3$). $^*P < 0.05$ *versus* control; $^{**}P < 0.01$ *versus* control; $^{\&}P < 0.05$ *versus* GB; $^{@}P < 0.05$ *versus* IP.

Discussion

This study presents the first data to show that GB is capable of preconditioning as mild ischaemia to protect cultured cortical neurons against ischaemia-induced apoptosis. Our findings provided the direct evidence that both GB and IP could protect neurons against ischaemia-induced apoptosis by similar pathway.

GB is an important active monomer of Ginkgolides, and has been shown to protect against neural damage such as attenuating hypoxic-ischaemic brain injury in immature rats, decreasing amyloid-beta damaged neuronal death and having beneficial effects on circulatory and inflammatory conditions [8, 10]. GB, as a competitive platelet-activating factor (PAF) receptor antagonist, is also designated to BN52021, which could inhibit intracellular Ca^{2+} increase induced by PAF in a concentration-dependent manner [8]. To date, there are no data available to show whether GB has

effects of preconditioning on the central nervous system and other tissues. An important observation of this work is that pre-treatment with GB or IP could significantly decrease the percentage of apoptosis and expression of active caspase-3 in ischaemic neurons. Our study showed for the first time that GB plays a key role of pharmacological preconditioning like IP in preventing neuronal apoptosis induced by ischaemia.

HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β [21]. Hypoxia causes an increase in HIF-1α by preventing its degradation in response to low O_2 tension, whereas the HIF-1β subunit is constitutively expressed [13, 21]. It is well known that HIF-1 and its target genes mediate critically hypoxia/ischaemia preconditioning in the brain [13, 20, 21]. Pre-treatment of neonatal rats with divalent cobalt chloride and iron chelating agent desferoxamine for 24 hrs before hypoxia/ischaemia afforded significant brain protection and increased HIF-1α content [13, 22]. The results of the present study showed that HIF-1α expression was also sensitive to GB in neurons, and that pre-treatment with GB, like IP, induced a significant increase in the content of HIF-1α and in cell viability in ischaemic neurons, implying that HIF-1 activation might also contribute to the GB-induced neuroprotective effect against ischaemic injury as is the case with IP in neuroprotection.

Hypoxia or ischaemia can strongly induce EPO, one of the most prominent HIF-1 targets [20, 23]. EPO was demonstrated recently to have a neurological function by preventing neuronal apoptosis after cerebral ischaemia through activation of extracellular signal-regulated kinases and protein kinase Akt-1/protein kinase B [24]. Application of EPO significantly increased survival and function of retinal ganglion cells in rats suffering from optic neuritis through increasing the protein levels of phospho-Akt, phospho-MAPK 1 and 2 and Bcl-2 [25]. EPO protected post-ischaemic hearts by preventing extracellular matrix degradation though JAK2-ERK pathway [26]. Tolerance to hypoxia-induced stroke in mice is mediated by EPO [23]. Therefore, we investigated the effects of GB on expression of EPO in this study. As was expected, a significant increase in EPO was found in ischaemic neurons pre-treated with GB or IP. Moreover, it was also found that the level of HIF-1α and EPO in non-ischaemic neurons treated only with GB or IP was higher than that in control. These results suggested that EPO not only had neuroprotective role in IP but might be one of the essential mediators in GB-inducced pharmacological preconditioning. However, it is unknown whether there are any other molecules associated with GB-induced pharmacological preconditioning besides HIF-1 and EPO, which needs our further study.

Bad is a key player in cell survival decisions and one of the BH3-only proapoptotic members, which is regulated post-translationally by several signalling networks and has been shown to be phosphorylated extensively on serine by kinases [27, 28]. Akt1 is one of these kinases that mediated the phosphorylation of Bad at Ser[136] [28–30]. Phosphorylation of Bad at Ser[136] has been demonstrated to inactivate its proapoptosis by sequestering Bad from Bcl-2 and/or Bcl-XL, leading to the survival of cells [28]. Our results showed that GB or IP induced a significant increase in the levels of [136]p-Bad, in company with a decrease in the apoptosis rate in ischaemic neurons. This finding implies that the anti-apoptotic role of GB or IP was mediated at least partly by Bad phosphorylation in ischaemic neurons.

It is well documented that the PI3K signalling pathways play a key role in the regulation of HIF-1α expression and content. HIF-1α protein level during hypoxic conditions is regulated by PI3K/Akt signalling pathway in HeLa cells [31, 32]. Our findings demonstrated that both GB and IP could significantly increase HIF-1α, EPO and [136]p-Bad, and pre-treatment with a specific PI3K inhibitor LY294002 significantly reduced the expression of EPO and [136]p-Bad induced by GB or IP, suggesting that enhanced expression of HIF-1α, EPO and [136]p-Bad induced by GB or IP might be mainly mediated by the PI3K/Akt/Bad pathways. EPO itself could also increase the level of Bad phosphorylation *via* PI3K/Akt pathway, which phosphorylates Bad at specific Ser[136] to prevent neurons from apoptosis [20]. Enhancement of p-Bad could induce the release of Bcl-2 from Bcl-2/Bad dimmer. Bcl-2 is generally believed to inhibit apoptosis by safeguarding mitochondrial membrane integrity and preventing the release of mitochondrial cytochrome c. Once released to the cytosol, cytochrome c could activate caspase-9 to initiate cysteine proteases cascade reaction [18, 28]. Our results showed that GB or IP could not only increase the expression of EPO and [136]p-Bad but also decrease the level of active caspase-3 protein, implying that GB might inhibit activation of caspase-3 by up-regulating the expression of EPO and enhancing Bad phosphorylation. In addition, our study found that GB or IP could induce a significant increase in expression of p-GSK-3β in ischaemic neurons, which further demonstrated that PI3K pathway could play a critical role in GB preconditioning. It was reported that GSK-3β can also inhibit activation of caspase-3 through PI3K/Akt/GSK-3β signalling pathway [18]. However, it should be pointed out that further studies are absolutely needed to understand whether there are any other signalling pathways to mediate the role of GB-induced pharmacological preconditioning.

In summary, our study suggests that GB is capable of preconditioning like IP against neuronal apoptosis induced by ischaemia by increasing the expression of HIF-1α, EPO and phosphorylated Bad through PI3K signalling pathway. The study also implies that GB might be a safe and effective agent in preventing cerebrovascular disease.

Acknowledgements

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