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Ginkgolides Protect PC12 Cells Against Hypoxia-Induced Injury by p42/p44 MAPK Pathway-Dependent Upregulation of HIF-1α Expression and HIF-1DNA-Binding Activity

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Abstract We hypothesized that the neuroprotective role of the standardized Ginkgo biloba (Ginkgoaceae) extract EGb 761 under hypoxic conditions might be associated with its function to increase HIF-1 activity based on the fact that oxygen availability is crucial for cellular metabolism and viability and that HIF-1 plays an essential role in cellular oxygen homeostasis under hypoxic conditions. In this study, we therefore investigated the effects of ginkgolides, the main constituent of the non-flavone fraction of EGb 761, on the content and activity of HIF-1α, a key factor to determine HIF-1 activity, in hypoxic PC12 cells induced by cobalt chloride. Our data demonstrated that ginkgolides have a significant protective role against hypoxia-induced injury in the PC12 cells. The findings also strongly support our hypothesis that the protective role of ginkgolides is due to the up-regulation of HIF-1α protein expression and modification through the ginkgolides-induced activation of the p42/p44 MAPK pathway. In addition, it was evident that ginkgolides could significantly increase the HIF-1 DNA binding activity, which might also be associated with the protective effects of ginkgolides by promoting the expression of target genes of HIF-1 under hypoxic conditions. J. Cell. Biochem. 103: 564–575, 2008.

Key words: neuroprotection; ginkgolides; extracellular signal-regulated kinase (ERK); HIF-1α expression and modification; activation of p42/p44 (ERK) MAPK pathway

Hypoxia-inducible factor-1 (HIF-1), a dimeric transcriptional activator, is now recognized as a master regulator of cellular and systemic oxygen homeostasis. It is expressed in many cell types [Semenza, 2001a,b; Lahiri et al., 2006]. This activator is composed of two subunits:

Abbreviations used: ARNT (HIF-1β), aryl hydrocarbon nuclear receptor translocator; CoCl2, cobalt chloride; ERK, extracellular signal-regulated kinase; HIF-1, hypoxia-inducible factor-1; HIF-1α, α unit of hypoxia-inducible factor-1; HRE, hypoxic response elements; MAPK, p42/p44-mitogen-activated protein kinase; MKPs, protein kinase phosphatases; NGF, nerve growth factor; p-ERK, phosphorylated ERK1/2; pVHL, prolyl hydroxylation von Hippel-Lindau tumor suppressor proteins; PHD, sprolyl hydroxylases; PMN, polymorphonuclear leukocyte; VEGF, vascular endothelial growth factor.

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HIF-1α and HIF-1β [Wang and Semenza, 1995; Wang et al., 1995; Jiang et al., 1996]. HIF-1β, also known as aryl hydrocarbon nuclear receptor translocator (ARNT), is constitutively expressed and unaffected by hypoxia or normoxia [Bunn and Poyton, 1996; Semenza, 2000]. HIF-1α is continuously synthesized and destroyed in normoxia as a result of its ubiquitination and subsequent degradation by the proteasomal system after hydroxylation [Wang and Semenza, 1995; Lahiri et al., 2006]. The HIF-1α half-life under normoxic conditions is less than 10 min and the protein is hardly detectable [Chun et al., 2002]. Under hypoxic conditions, however, HIF-1α prolyl hydroxylases (PHDs) are inactive which prevents the binding of prolyl hydroxylation von Hippel-Lindau tumor suppressor proteins (pVHL). Therefore, HIF-1α escapes ubiquitination and proteasomal degradation, resulting in the protein accumulation and dimerization with HIF-1β [Jewell et al., 2001]. The heterodimer can then bind to hypoxic response elements (HRE)-containing promoter regions in target genes and increase their expression [Wang and Semenza, 1995; Huang et al., 1998; Kallio et al., 1999].

Over 70 genes operated in all cells are directly regulated by HIF-1 in response to hypoxia [Semenza, 2003]. The proteins encoded by these genes mediate adaptive physiological responses, such as angiogenesis, erythropoiesis, and glycolysis that either serve to increase O_2 delivery or allow metabolic adaptation to reduced O_2 availability [Lahiri et al., 2006], and also regulate several important polymorphonuclear leukocyte (PMN) functions relevant to the host defense [Peyssonnaux et al., 2005; Zarember and Malech, 2005]. The increase in the expression of glycolytic enzymes, the glucose transporter-1, or the vascular endothelial growth factor (VEGF) all contribute towards the survival of cells and tissues undergoing oxygen deficiency [Semenza, 2002, 2003]. In the process of HIF-1 activation by hypoxia, phosphorylation of the HIF-1α subunit is crucial for this adaptative response [Lahiri et al., 2006].

Under hypoxic conditions, it has also been reported that the standardized Ginkgo biloba (Ginkgoaceae) extract EGb 761 has neuroprotective effects in different models in vivo and in vitro [Smith et al., 1996; Zhu et al., 1997a,b; Bastianetto and Quirion, 2002; Luo et al., 2002; Ahlemeyer and Krieglstein, 2003a,b; Zhu et al., 2004]. Ginkgo biloba is an ancient Chinese tree and the extracts from its leaves have been used in Chinese herbal medicine for thousands of years and shown to exert a wide range of biological activities. However, studies on the biochemical and biomedical effects of Ginkgo biloba extracts are still at a very early stage [Smith et al., 1996; Maclennan et al., 2002]. A number of key questions have not yet been answered. Although there is substantial experimental evidence to support that the flavonoid fraction [Bastianetto et al., 2000] and non-flavone fraction [Liu et al., 1996; Sharma et al., 2000] of EGb 761 may contribute to the neuroprotective properties of the Ginkgo biloba leaf, it is unknown which of the compounds of EGb 761 mediates its neuroprotective activity. Also, the relevant mechanisms involved in the neuroprotection are not known in detail.

Published studies have demonstrated that the non-flavone fraction was responsible for the antihypoxic activity of EGb 761 [Maclennan et al., 2002; Ahlemeyer and Krieglstein, 2003a,b]. In addition, we speculated that the neuroprotective role of EGb 761 under hypoxic conditions might be associated with its function to increase HIF-1 activity based on the fact that oxygen availability is crucial for cellular metabolism and viability and that HIF-1 is the major oxygen homeostasis regulator which plays an essential role in cellular oxygen homeostasis under hypoxic conditions [Chandel and Schumacker, 2000]. In this study, we therefore investigated the effect of ginkgolides (Gins A, B, C and J), the main constituent of the non-flavone fraction of EGb 761, on the changes in content and activity of HIF-1α in the hypoxic PC12 cells induced by cobalt chloride ($CoCl_2$) and the relevant mechanisms involved.

The PC12 cell was chosen for this study as a neuronal model because it can be differentiated into neuron following the treatment of nerve growth factor and also because it is oxygen-sensitive [Czyzyk-Krzeska et al., 1994; Norris and Millhorn, 1995]. $CoCl_2$, a water-soluble compound, was used in this investigation because it is the best-known chemical inducer of hypoxia-like responses such as erythropoiesis and angiogenesis in vivo [Bergeron et al., 2000; Piret et al., 2002]. We chose to study HIF-1α because the regulation of HIF-1 activity mostly depends on its α subunit [Lahiri et al., 2006]. Our findings of cell viability and morphological observation confirm that ginkgolides has a

significantly protective role in the PC12 cells against injury induced by $CoCl_2$. The data obtained also indicated that the neuroprotective role of ginkgolides against hypoxia-induced injury is mediated by the up-regulation of HIF-1α expression, the modification and DNA-binding activity through the activation of the p42/p44 (ERK) MAPK pathway.

MATERIALS AND METHODS

Reagents

Nerve growth factor (NGF) was purchased from Alomone Labs Ltd., Jerusalem, Israel and ginkgolides (95.35%: ginkgolide-A 30%, ginkgolide-B 56%, Gginkgolide-C 3%, ginkgolide-J 0.5–1%) were obtained from China Pharmaceutical University. Genistein, PD 98059, cobalt chloride ($CoCl_2$), poly-L-lysine and anti-β-actin antibody were bought from Sigma Chemical Company, St. Louis, MO and anti-phosphorylated ERK (extracellular signal-regulated kinase)1/2 polyclonal antibody (Thr202/Tyr204 and anti-total ERK1/2 polyclonal antibody were from Cell Signaling Technology, Beverly, MA. Anti-HIF-1α monoclonal antibody was purchased from Novus Biologicals, Littleton, CO and secondary antibodies (HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG) were from Pierce, Rockford, IL.

Cell Culture

The rat pheochromocytoma PC12 cells, purchased from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in a poly-L-lysine coated 25 cm^2 flask (Corning) in RPMI 1,640 medium supplemented with 5% fetal bovine serum, 10% horse serum (Gibco), 100 units/ml penicillin and 100 g/ml streptomycin in a 5% CO_2 incubator (NAPCO 5400) at 37°C. The cells were suspended and forced 10 times through a needle to form single-cell suspension. After the PC12 cells were grown to 80% confluence in the flask, the cells were treated by NGF (final concentration was 0.05 (g/ml) and seeded in 96-well plates precoated with poly-L-lysine at 1×10^4 cells per well. The PC12 cells were then maintained in DMEM medium supplemented with 3% fetal bovine serum and 3% horse serum in the density of 1×10^6 cells/ml after the treatment with NGF. The medium was replaced by non-serum DMEM containing 37.5 μg/ml ginkgolides or other concentrations and then treated with 125 (mol/L of $CoCl_2$ for 4 h. To investigate the signal pathway involved, PD98059 (100 (mol/L) or genistein (150 (mol/L) was added to the medium at 1 h before the treatment with $CoCl_2$.

Determination of Cell Viability

Neuronal viability was assessed using a MTT assay as described by Jiang et al. [1996] in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in live cells. Briefly, a total of 5 mg/ml MTT was added to each well (final concentration is 1 mg/ml) and another 4 h of incubation at 37°C was conducted. The assay was stopped by the addition of a 100 (l lysine buffer (20% SDS in 50% *N*′,*N*-dimethylformamide, pH 4.7). Optical density (OD) was measured at 570 nm by the use of the ELX-800 microplate assay reader.

Western Blot Analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in a 100–200 μl cold lysis buffer (10 mmol/L Tris–HCl with pH 7.6, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 2 mmol/L DTT, 0.4 mmol/L PMSF, 2 mg/L aprotinin, 2 mg/L leupeptin, and 2 mg/L pepstain). Lysates were kept in ice for 30 min, sonicated for 10 s using Soniprep 150 (MSE Scientific Instruments, England). After centrifugation at 10,000*g* for 15 min at 4°C, the supernatant was collected and the protein content was determined using the Bradford assay kit (Bio-Rad, Hercules, CA). The proteins were separated by 10% SDS-polyacrylamide gels, transferred to the PVDF membranes (Bio-Rad) previously blocked with 5% non-fat milk in TBS-T (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and incubated overnight at 4°C with the indicated primary antibodies and then incubated with the secondary antibodies for 2 h at room temperature. Immunolabeling was detected by the enhanced chemiluminiscence (ECL western blotting analysis system kit, Amersham Biosciences, England) and exposed to x-ray film (Kodak). The intensity of each band was scanned and quantified with Shine-tech Image System (Shanghai, China). Normalized values are presented.

Reverse Transcriptase-Polymerase Chain Reaction

TRIzol reagent (GIBCO) was used to extract the total cellular RNA from the confluent cells grown in 25-mm tissue culture plates according to the manufacturer's instructions. The total RNA was then quantified and the integrity was tested by gel electrophoresis. The total RNA (3 μg) from each sample was retrotranscripted to cDNA using the reverse transcription kit (GIBCO). PCR amplification for the cDNA of HIF-1α was carried out using the following primers: forward primer, 5′-CCCAAAGACAA-TAGCTTCGC-3′; reverse primer 5′-CTGCCTT-GTATGGGAGCATT-3′. All results were normalized with GAPDH primer forward primer, 5′-ACCACAGTCCATGCCATCAC-3′; reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′.

Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assay (EMSA)

The nuclear extracts of the cells treated with or without ginkgolides were prepared as described by Semenza and Wang [1992]. Electrophoretic mobility shift assay was performed according to the user's manual (Panomics' EMSA Kits AY1107). The sense and antisense strands for the HIF-1α binding sites in the promoter region of the erythropoietin gene were included in the kit. The sequence of the biotin labeled HIF-1α probe is 5′-AGCTTGCCC-TACGTGCTGTCTCAGA-3′, and the unlabeled nonsense oligonucleotides is 5′-GAGGAGGG-CTGCTTGAGGAAGTATAAGAAT-3′. For competition experiments, a 66-fold excess of the unlabeled HIF-1α probe was added before the addition of the labeled probe. For non-competition experiments, a 66-fold excess of the nonsense oligonucleotides was added. The products were loaded onto a 4% native polyacrylamide gel and run at a low voltage, and transferred to a nylon membrane for 30–45 min at 300 mA. After the transfer, bake the membrane for 1 h at 80°C in a dry oven. Biotin-labeled probe were detected by Streptavidin-HRP conjugate and enhanced chemiluminiscence.

Statistical Analysis

The statistical analyses were performed using SPSS 10.0. Data are presented as mean ± SEM. 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 Ginkgolides on the Viability and Morphology of PC12 Cells Treated With $CoCl_2$

The viability of the PC12 cell was determined using a MTT assay. To investigate the effects of ginkgolides on the viability of the PC12 cells that were treated with $CoCl_2$, the cells were pretreated with ginkgolides (37.5 μg/ml) for 24 h before the $CoCl_2$ treatment was conducted. The MTT assay results, as shown in Figure 1A, indicated that the treatment of the cells with 125 μmol/L of $CoCl_2$ for 4 h (hypoxic PC12 cells) led to a significant decrease in the cell viability, being only 59% of the control. However, the cell viability was significantly higher in the hypoxic PC12 cells pretreated with ginkgolides (37.5 μg/ml) than without ginkgolides (200% of the value of the hypoxic PC12 cells). The reduction in the viability of the PC12 induced by the $CoCl_2$

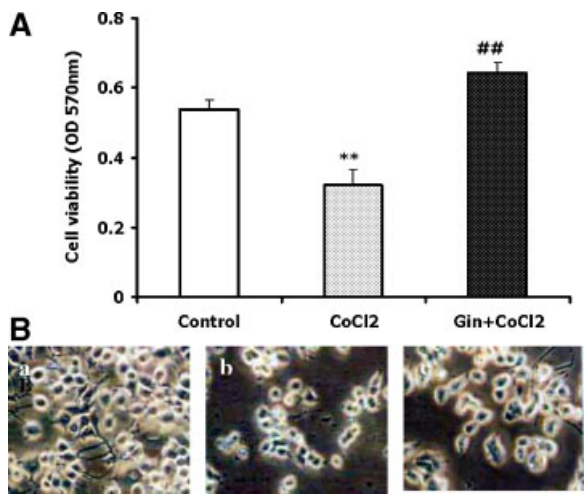

Fig. 1. Effects of ginkgolides on the viability and morphology of hypoxic PC12 cells. The PC12 cells were treated with ginkgolides (Gin, 37.5 μg/ml) for 24 h and then exposure to $CoCl_2$ (125 μmol/L) for 4 h. **A**: Effects of ginkgolides on the viability of hypoxic cells. Data were Mean ± SEM (n = 8). $^{**}P < 0.01$ versus the control, $^{\#\#}P < 0.01$ versus the $CoCl_2$ group. **B**: Representative phase-contrast micrographs of hypoxic cells. **a**: The control group: PC12 cells showing neuron-like morphology and stop proliferation and adherent-grown cells have phase-bright appearance with neuritis to form network. **b**: The $CoCl_2$ group: treated cells undergo asynchronous cell degeneration and some of the cell bodies shrinking with fragment neuritis. **c**: The Gin + $CoCl_2$ group: the cells showed no or slight injury both in bodies and in neuritis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treatment was completely recovered by the pretreatment of ginkgolides, implying a protective role of ginkgolides against the injury induced by $CoCl_2$ in the PC12 cells. In agreement with the results of cell viability, treatment of 125 μmol/L of $CoCl_2$ led to a significantly morphological change in the PC12 cells, showing asynchronous cell degeneration and some of the cell bodies shrinking with fragment neuritis (Fig. 1B). The cultures treated with 37.5 μg/ml ginkgolides for 24 h before exposure to $CoCl_2$ showed almost no injury both in bodies and in neuritis. The morphological observation also confirms that ginkgolides has a significantly protective role in the PC12 cells again the injury induced by $CoCl_2$.

Effects of Ginkgolides on the Content of HIF-1α in PC12 Cell Treated With $CoCl_2$

To further investigate the potential mechanisms involved in the protective role of ginkgolides in the PC12 cells against the injury induced by $CoCl_2$, we then examined the effect of ginkgolides on the content of HIF-1α in the PC12 cell treated with $CoCl_2$. Western blot results showed that the content of HIF-1α was significantly higher in the cells treated with $CoCl_2$ than that in the control cells (Fig. 2AB(E)). The treatment of the hypoxic PC12 cells with ginkgolides led to a further enhancement in the level of HIF-1α (Fig. 2AB(D)). The level of HIF-1α was significantly higher in the hypoxic PC12 cells treated with ginkgolides than that in the cells treated without ginkgolides.

When the cells were pre-incubated with 150 (mol/L of genistein (a nonspecific tyrosine kinase inhibitor) for 1 h before the treatment of $CoCl_2$, it was found that the increased content of HIF-1α, induced by the treatment of $CoCl_2$, could be completely inhibited by the pretreatment of genistein (Fig. 2AB(C)), while pretreatment with ginkgolides (37.5 (g/ml) for 24 h could significantly inhibit the reduction in HIF-1α level induced by the treatment of genistein (Fig. 2AB(B)), implying that the inhibiting effect of genistein on HIF-1α in the hypoxic PC12 cells could be blocked by ginkgolides. Similar results were also observed with the use of 100 (mol/L of PD98059 (an inhibitor of p42/44 MAPK or ERK) for 1 h before the treatment of $CoCl_2$. The increased HIF-1α level, induced by the treatment of $CoCl_2$, was found to be significantly inhibited by the pretreatment of

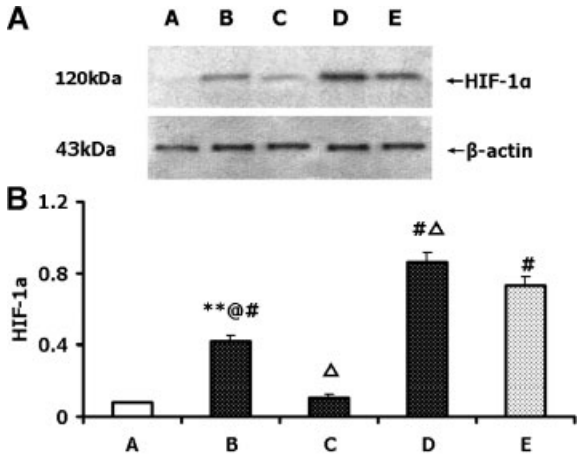

Fig. 2. Effects of ginkgolides on the content of HIF-1α in hypoxic PC12 cells. The PC12 cells were treated with ginkgolides (37.5 μg/ml) for 24 h and then expose to $CoCl_2$ (125 μmol/L) for 4 h. In some experiments, the cells were also preincubated with 150 (mol/L of genistein (a nonspecific tyrosine kinase inhibitor) for 1 h before incubation with $CoCl_2$. **A**: A representative experiment of Western blot of HIF-1α and β-actin. A: The control; B: Ginkgolides + Genistein + $CoCl_2$; C: Genistein + $CoCl_2$; D: Ginkgolides + $CoCl_2$; E: $CoCl_2$ only. **B**: Quantification of expression of HIF-1α protein in PC cells. Expression values were normalized for β-actin and the data were presented as Mean ± SEM (n = 3). $^{**}P < 0.01$ versus D (Ginkgolides + $CoCl_2$); $^{\Delta}P < 0.01$ versus E ($CoCl_2$); $^{@}P < 0.01$ versus C (Genistein + $CoCl_2$); $^{\#}P < 0.01$ versus A (The control).

genistein. Also, pretreatment with ginkgolides (37.5 (g/ml) for 24 h could significantly inhibit the reduction in HIF-1α induced by the treatment of PD98059.

Effects of Ginkgolides on the Activity of Extracellular Signal-Regulated Kinase (ERK) in PC12 Cell Treated With $CoCl_2$

To find out whether the increased content of HIF-1α protein induced by ginkgolides is associated with the p42/p44-mitogen-activated protein kinase (MAPK) pathway, we investigated the effects of ginkgolides on the activity of the extracellular signal-regulated kinase (ERK), a member of the MAPK family, in the PC12 cells treated with $CoCl_2$. The data presented in Figure 3 showed that phosphorylated ERK1/2 (p-ERK) was significantly higher in the cells treated with $CoCl_2$ than in the control cells (Fig. 3ABCD(E)). Same as the findings of the effect of ginkgolides on HIF-1α, the treatment with ginkgolides also led to a further enhancement in the activity of p-ERK in the hypoxic PC12 cells (Fig. 3ABCD(D)). The p-ERK was

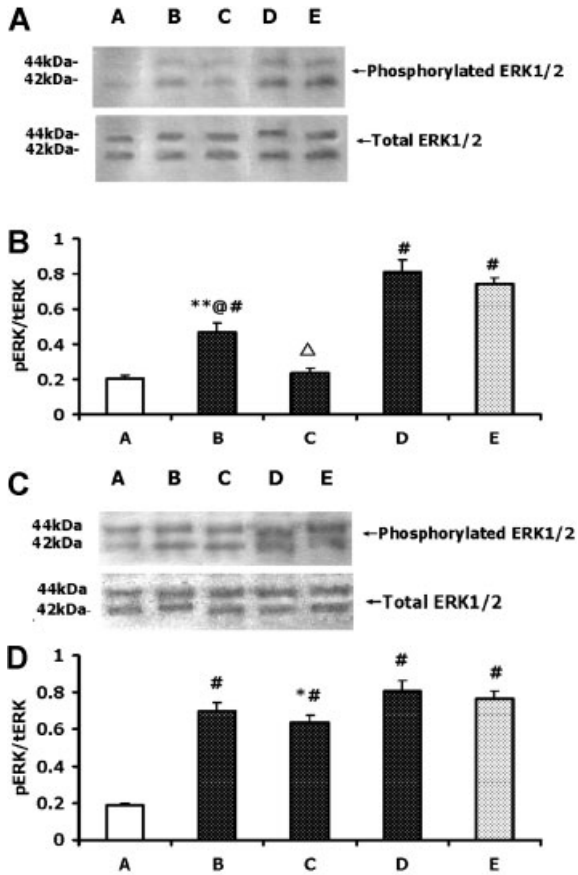

Fig. 3. Effects of ginkgolides on the activity of extracellular signal-regulated kinase (ERK) in PC12 cells. The PC12 cells were treated with ginkgolides (37.5 μg/ml) for 24 h and then incubated with or without 100 (mol/L of PD98059 or 150 (mol/L of genistein for 1 h before incubating with $CoCl_2$ (125 μmol/L) for 4 h. **A**: A representative experiment of Western blot of phosphorylated ERK1/2 (PD98059). The bands on the gel correspond to the expected molecular weights of phosphorylated ERK1/2. A: The control; B: Ginkgolides + PD98059 + $CoCl_2$; C: PD98059 + $CoCl_2$; D: Ginkgolides + $CoCl_2$; E: $CoCl_2$ only. **B**: Quantification of expression of phosphorylated ERK1/2 bands compare to that of total ERK1/2 in PC cells (PD98059). The data were presented as Mean ± SEM (n = 3). $^{**}P < 0.01$ versus D (Ginkgolides + $CoCl_2$); $^{\Delta}P < 0.01$ versus E ($CoCl_2$); $^{@}P < 0.01$ versus C (PD98059 + $CoCl_2$); $^{\#}P < 0.01$ versus A (The control). **C**: A representative experiment of Western blot of phosphorylated ERK1/2 (Genistein). A: The control; B: Ginkgolides + Genistein + $CoCl_2$; C: Genistein + $CoCl_2$; D: Ginkgolides + $CoCl_2$; E: $CoCl_2$ only. **D**: Quantification of expression of phosphorylated ERK1/2 bands compare to that of total ERK1/2 in PC cells (Genistein). The data were presented as Mean ± SEM (n = 3). $^{*}P < 0.05$ versus D (Ginkgolides + $CoCl_2$); $^{\Delta}P < 0.01$ versus E ($CoCl_2$); $^{@}P < 0.01$ versus C (Genistein + $CoCl_2$); $^{\#}P < 0.01$ versus A (The control).

significantly higher in the hypoxic PC12 cells treated with ginkgolides than that in the cells treated without ginkgolides.

Pretreatment of the hypoxic PC12 cells with PD98059 (100 (mol/L) led to a significant reduction in p-ERK (Fig. 3AB(C)), being about only 25% of the hypoxic cells treated without PD98059 (Fig. 3AB(E)). This implied that PD98059 (100 (mol/L) could almost completely inhibit the $CoCl_2$-induced activation of ERK. It was also found that the inhibition of PD98059 on p-ERK activities in the $CoCl_2$-treated cells could be partly inverted by ginkgolides. Pretreatment with ginkgolides resulted in a twofold increase in p-ERK (Fig. 3AB(B)) in the $CoCl_2$-treated cells pretreated with PD98059 (Fig. 3AB(B,C)). The levels of p-ERK in the hypoxic PC12 cells pretreated with ginkgolides and PD98059 (Fig. 3AB(B)) were still lower than those in the hypoxic PC12 cells that received no treatment (Fig. 3AB(E)). In addition, genistein (150 (mol/L) significantly inhibited the activation of ERK in the cells treated with $CoCl_2$. It was found that the increased p-ERK, induced by the treatment of $CoCl_2$, was significantly inhibited by pretreatment of genistein (150 (mol/L) (Fig. 3CD(C)). Also, pretreatment with ginkgolides (37.5 (g/ml) could significantly revise the inhibition on p-ERK induced by genistein (Fig. 3CD(B)), implying that the inhibiting effect of genistein on p-ERK in the hypoxic PC12 cells could be partly blocked by ginkgolides.

Effects of Ginkgolides on HIF-1α mRNA Expression in PC12 Cell Treated With $CoCl_2$

To determine whether there is a transcriptional regulation of ginkgolides on the expression of HIF-1α, we investigated the effects of ginkgolides on the levels of HIF-1α mRNA in the PC12 cells. The cells were preincubated with or without ginkgolides (37.5 μg/ml) for 24 h and then exposed to $CoCl_2$ for 4 h. In some experiments, the cells were treated only by incubation with ginkgolides (37.5 μg/ml) for 24 h. The measurements of the mRNA level by RT-PCR method showed that there was a significant increase in HIF-1α mRNA in the cells treated with only $CoCl_2$ or ginkgolides as compared with the control cells (Fig. 4). It was also found that the level of HIF-1α mRNA in the cells pretreated with ginkgolides was significantly higher than that in the cells pretreated without ginkgolides under hypoxic conductions (Fig. 4). The data imply that ginkgolides has a role to increase the transcription of HIF-1α gene under not only hypoxic but also normoxic conditions.

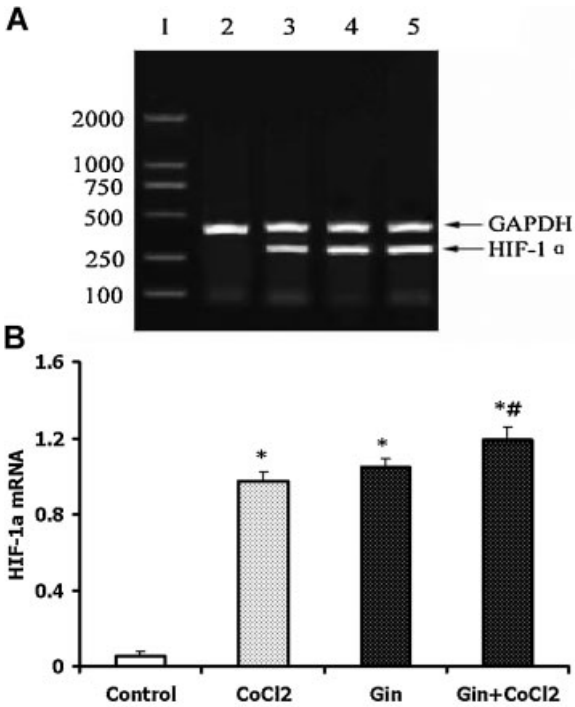

Fig. 4. Effects of ginkgolides on HIF-1α mRNA expression in PC12 cells. The cells were preincubated with or without ginkgolides (Gin, 37.5 μg/ml) for 24 h and then exposed to $CoCl_2$ for 4 h. In some experiments, the cells were treated only by incubation with ginkgolides (37.5 μg/ml) for 24 h without the further treatment with $CoCl_2$. **A**: A representative experiment of RT-PCR products of HIF-1α and GAPDH mRNA. The bands on the gel correspond to the expected oligonucleotide size based on the primers used for HIF-1α and GAPDH. 1: DNA marker; 2: the control; 3: $CoCl_2$; 4: Gin; 5: Gin + $CoCl_2$; **B**: Expression of HIF-1α mRNA was quantified by normalization to GAPDH mRNA and the data were presented as Mean ± SEM (n = 3). $^{**}P < 0.01$ versus the control; $^{\#}P < 0.01$ versus $CoCl_2$.

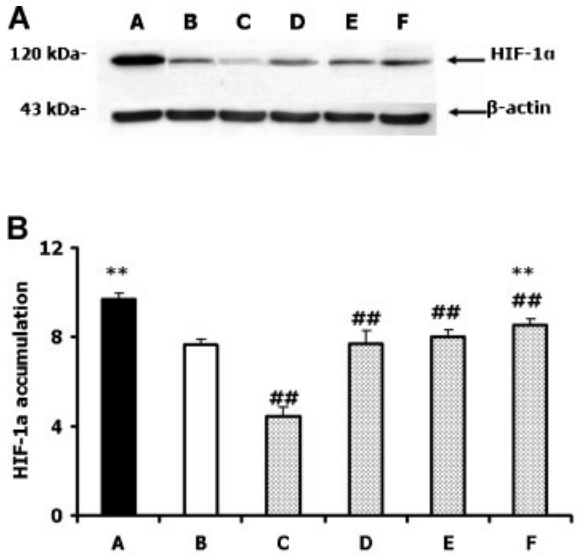

Fig. 5. Effects of ginkgolides on HIF-1α accumulation and HIF-1 DNA binding activity in PC12 cells. The PC12 cells were incubated with different concentrations of ginkgolides (0, 9.75, 18.75, 37.5 (g/ml) for 24 h and whole cell lysates (60 μg of protein) were then prepared and analyzed by Western blotting using antibodies to HIF-1α. In some experiments, the cells were pre-incubated with 150 (mol/L of genistein or 100 (mol/L of PD98059 for 1 h and then incubated with 37.5 (g/ml of ginkgolides before Western blotting analysis. **A**: A representative experiment of Western blot of HIF-1α and β-actin. Lane A: 37.5 (g/ml of ginkgolides for 24 h; Lane B: the control; Lane C: 150 (mol/L Genistein for 1 h + 37.5 (g/ml of ginkgolides for 24 h; Lane D: 100 (mol/L PD98059 for 1 h + 37.5 (g/ml of ginkgolides for 24 h; Lane E: 9.37 (g/ml of ginkgolides for 24 h; Lane F: 18.75 (g/ml of ginkgolides for 24 h. **B**: Quantification of HIF-1α protein accumulation in PC12 cells. Values were normalized for β-actin and the data were presented as Mean ± SEM (n = 3). $^{**}P < 0.01$ versus the control (Lane 2), $^{\#\#}P < 0.01$ versus 37.5 (g/ml of ginkgolides for 24 h (Lane 1).

Effects of Ginkgolides on HIF-1α Accumulation and HIF-1 DNA Binding Activity in PC12 Cells

Finally, the effects of ginkgolides on HIF-1α accumulation and HIF-1 DNA binding activity were investigated. The PC12 cells were incubated with different concentrations of ginkgolides (0, 9.75, 18.75, 37.5 (g/ml) for 24 h and Western blotting analysis was then conducted. The results presented in Figure 5 showed that treatment with ginkgolides at 18.75 and 37.5 (g/ml for 24 h could significantly stimulate HIF-1α protein accumulation in the PC 12 cells (Line A: 37.5 (g/ml, Line B: 0 (g/ml, Line E: 9.75 (g/ml and Line F: 18.75 (g/ml). When the cells was pre-incubated with 150 (mol/L of genistein (Line C) or 100 (mol/L of PD98059 (Line D) for 1 h before the addition of 37.5 (g/ml of ginkgolides, HIF-1α accumulation was found to be significantly inhibited ($P < 0.01$ vs. Line 1). Further, the data in Figure 6 indicated that ginkgolides treatment could significantly stimulate HIF-1 DNA binding activity. The increased HIF-1 DNA binding activity induced by ginkgolides suggested a role of ginkgolides to promote the expression of the target genes of HIF-1 which might be also associated with the protective effects of ginkgolides under hypoxic conditions because the proteins encoded by these target genes of HIF-1 mediated a number of adaptive physiological responses.

DISCUSSION

The PC12 cell, a rat pheochromocytoma cell line derived from a tumor of adrenal medulla chromaffin tissue, is an oxygen-sensitive cell type that provides a very useful system to

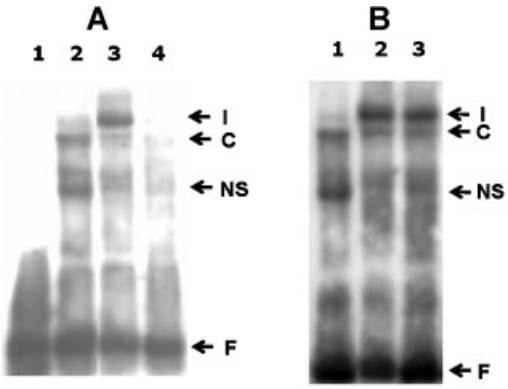

Fig. 6. Effects of ginkgolides on HIF-1 DNA binding activity in PC12 cells. Nuclear extracts (5 μg of protein) were incubated with the labeled transcription factor Probe (TF probe: the biotin-labeled transcription factor DNA probes) containing the hypoxia response element from the erythropoietin promoter. **A**. Competition experiments: Lane 1: free TF probes without nuclear extract; Lane 2: TF probes with nuclear extract from the PC12 cells; Lane 3: TF probes with nuclear extract from the PC12 cells pretreated by 37.5 (g/ml of ginkgolides; Lane 4: special competition of ginkgolides TF probes with nuclear extract from the PC12 cells pretreated by 37.5 (g/ml of ginkgolides and 66-fold unlabeled special oligonucleotide probes (cold probes). Band I: inducible binding activities; Band C: constitutive binding; Band NS: nonspecific binding; Band F: free probes. Experiments shown are representative of experiments performed three times with identical results. **B**. Non-competition experiments: Lane 1: TF probes with nuclear extract from the PC12 cell; Lane2: TF probes with nuclear extract from the PC12 cells pretreated by 37.5 (g/ml of ginkgolides; Lane 3: Nonspecial competition of ginkgolides TF probes with nuclear extract from the PC12 cell pretreated by 37.5 (g/ml of ginkgolides and 66-fold unlabeled nonspecial oligonucleotide probes (cold probes). The competition and non-competition experiments indicated that the stimulation of ginkgolides on HIF-1 DNA binding activity is specific.

conduct studies on hypoxia at the cellular level. Some studies [Czyzyk-Krzeska et al., 1994; Norris and Millhorn, 1995] have demonstrated that a very small reduction in atmospheric oxygen can dramatically induce a change in the expression and mRNA stability of some genes. Thus, these cells were used in this study. Our data showed that treatment of the PC12 cells with 125 μmol/L of $CoCl_2$ resulted in a 41% decrease in the cell viability. However, the reduction in the viability of PC12 induced by $CoCl_2$ was completely recovered by pretreatment of ginkgolides. In agreement with the results of cell viability, treatment of $CoCl_2$ led to a significantly morphological change in the PC12 cells, whereas pretreatment with ginkgolides before exposure to $CoCl_2$ showed almost no injury both in bodies and neuritis of the cells. These findings provide a solid evidence for the existence of a protective role of ginkgolides against injury induced by $CoCl_2$ or hypoxia in the PC12 cells.

Since oxygen availability is crucial for cellular metabolism and viability and that HIF-1 is the major oxygen homeostasis regulator which plays an essential role in cellular oxygen homeostasis under hypoxic conditions, functioning as a widely operative transcriptional control system responding to physiological levels of cellular hypoxia [Iyer et al., 1998; Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Semenza, 2002, 2003], we hypothesized that ginkgolides might have a potential effect on the HIF-1 activity. The changes in the HIF-1 activity induced by ginkgolides might be one of the mechanisms associated with its neuroprotective role in the hypoxic PC12 cells. Since the regulation of HIF-1 activity depended mostly upon the α subunit, we then investigated the effects of ginkgolides on the content of HIF-1α in the PC12 cell treated with $CoCl_2$. Western blot results confirmed that HIF-1α level was significantly higher in the cells treated with $CoCl_2$ than that in the control cells and also the treatment of ginkgolides induced a further enhancement in the HIF-1α content in the hypoxic PC12 cells. The level of HIF-1α protein was significantly higher in the hypoxic PC12 cells treated with ginkgolides than that in the cells treated without ginkgolides. The findings support our hypothesis that the protective role of ginkgolides against injury induced by $CoCl_2$ or hypoxia in the PC12 cells is at least partly associated with the up-regulation of the HIF-1α protein and then the increased HIF-1 activity (Fig. 7).

Our findings also showed that pretreatment with PD98059 (an inhibitor of p42/44 MAPK or ERK) or genistein (a nonspecific tyrosine kinase inhibitor) could significantly inhibit the increased HIF-1α protein content induced by $CoCl_2$, while ginkgolides could significantly reduce this inhibition induced by PD98059 or genistein on the HIF-1α protein. It implied that the inhibiting effect of PD98059 or genistein on HIF-1α protein in the hypoxic PC12 cells could be blocked by ginkgolides. It led us to propose that ginkgolides might have a role to activate the p42/p44 MAPK pathway and that the increased content of the HIF-1α protein induced by ginkgolides might be at least partly mediated by the activation of the p42/p44 MAPK pathway

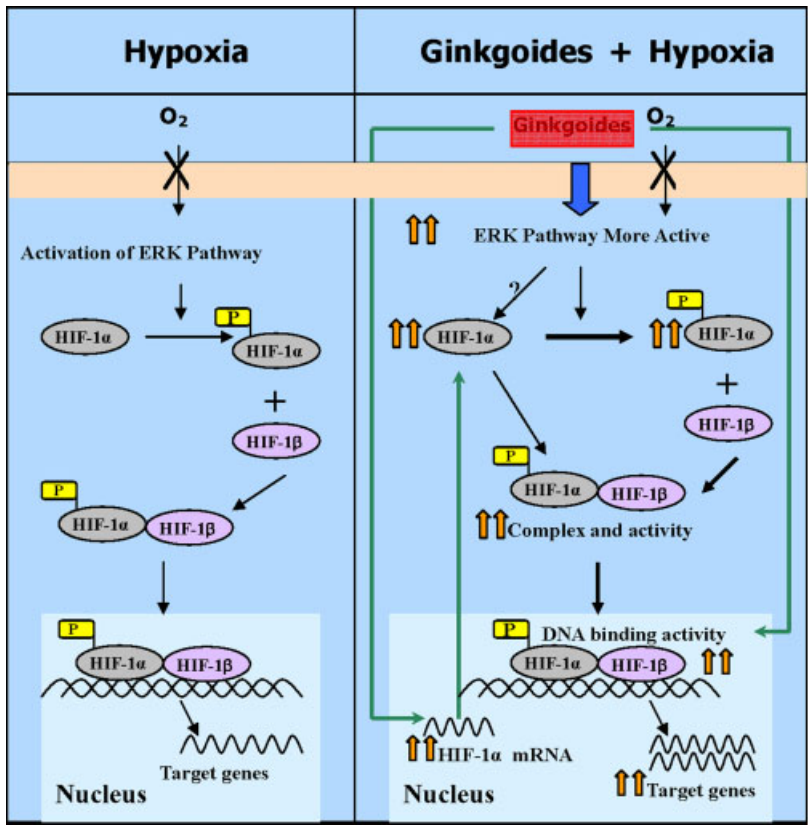

Fig. 7. Hypothesized mechanisms for the neuroprotective role of ginkgolides. Ginkgolides might have a role to up-regulate the HIF-1α protein expression by its direct effect on the HIF-1α mRNA translation and/or promoting HIF-1α mRNA transcription, and to stimulate HIF-1α phosphorylation or modification through the activation of the p42/p44 MAPK pathway. In addition, ginkgolides might have the ability to increase the HIF-1 DNA binding activity directly, which might also be associated with the protective role of ginkgolides by promoting the expression of the target genes of HIF-1 under hypoxic conditions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that had been demonstrated to play an important role in mediating neuroprotective adaptations in a rat model [Shamloo et al., 1999]. We further investigated the effects of ginkgolides on the activity of the extracellular signal-regulated kinase (ERK), a member of the MAPK family, in the PC12 cells treated with $CoCl_2$. Our data demonstrated that ginkgolides could induce a significant increase in the content of the p-ERK (phosphorylated ERK1/2) in the hypoxic PC12 cells. The findings also showed that the increased content of p-ERK induced by hypoxia could be almost completely inhibited by PD98059, whereas this inhibition could be significantly (although not completely) inverted by ginkgolides. In addition, genistein significantly inhibited the activation of p-ERK in the hypoxic cells, also, ginkgolides could significantly revise this inhibition. The fact that ginkgolides have a direct role to increase p-ERK content as well as to revise significantly the inhibiting effects of PD98059 and genistein on p-ERK in the hypoxic cells provide strong evidence to support our hypothesis that the increased content of the HIF-1α protein is at least partly mediated by the ginkgolides-activated p42/p44 MAPK pathway in the hypoxic PC12 cells (Fig. 7). The p42/p44 MAPK pathway activated by ginkgolides might play a key role in the up-regulation of the HIF-1α expression and modification in the PC12 cells under our experimental conditions.

The HIF-1 DNA binding activity is another important factor on that the transcriptional activity of the HIF-1 complex is dependent. The increased HIF-1 DNA binding activity will enhance the expression of the target genes of HIF-1 and then increase the protective adaptive responses for the cells to hypoxia [Semenza, 2002, 2003]. The results obtained in the

present study also showed that treatment with ginkgolides at higher concentrations could significantly stimulate the HIF-1α protein accumulation and HIF-1 DNA binding activity, and revise the inhibiting effects of genistein and PD98059. This evidences that ginkgolides have a role to promote the expression of the target genes of HIF-1 by its direct effect on HIF-1 DNA binding activity which might also be associated with the protective effects of ginkgolides under hypoxic conditions in addition to the up-regulation of the HIF-1α expression and modification in the hypoxic PC12 cells.

A number of studies demonstrated that the active MAPK family members could be rapidly inactivated through dephosphorylation by phosphatases known as the dual specificity mitogen-activated protein kinase phosphatases (MKPs) [Keyse, 2000; Slack et al., 2001; Stawowy et al., 2003] Among these phosphatases, MKP-1, encoded by an immediate early gene, showed an equal efficacy in dephosphorylating all the three MAPK isoforms [Keyse, 2000; Slack et al., 2001]. Fan and his colleagues [Liu et al., 2003, 2005] demonstrated that MKP-1 was implicated in the negative regulation of the HIF-1a subunit phosphorylation and HIF-1 activity. They also showed that hypoxia transcriptionally induced the MKP-1 expression in a time-dependent manner and also activated ERK. The activity of ERK was enhanced or reduced by the MKP-1 suppression or MKP-1 overexpression, respectively. Their findings indicated that the hypoxia-induced MKP-1 protected the overactivation of HIF-1 through the inhibiting activity of ERK. In this study, we did not investigate the effects of ginkgolides on the MKP-1 expression in the hypoxic cells. It is unknown whether ginkgolides have an inhibiting role in the MKP-1 expression and whether the increased expression of ERK induced by ginkgolides is partly mediated by the MKP-1 suppression by ginkgolides in the hypoxic PC12 cells. However, the possibilities are worth further investigation. In addition to MKP-1, the process of HIF-1 activation is mediated by several different protein kinase pathways. Further studies on the effects of ginkgolides on these pathways are also needed.

In summary, we reported for the first time that ginkgolides, the main constituent of the non-flavone fraction of EGb 761, have a significant protective role against the hypoxia-induced injury in the PC12 cells. The findings obtained strongly supported our hypothesis that the protective role of ginkgolides is at least partly due to the up-regulation of the HIF-1α protein expression and modification through the ginkgolides-induced activation of the p42/p44 MAPK pathway (Fig. 7). Our data also demonstrated that ginkgolides could significantly increase the HIF-1 DNA binding activity, which might also be associated with the protective effects of ginkgolides under hypoxic conditions by promoting the expression of the target genes of HIF-1. The results also implied a potential for ginkgolides to be developed into a new effective drug for clinical use to protect neuron or cell under different pathological conditions.

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