Activation of calcium/Calmodulin-Dependent Protein Kinase IV and Peroxisome Proliferator-Activated Receptor γ Coactivator-1α Signaling Pathway Protects Against Neuronal Injury and Promotes Mitochondrial Biogenesis in the Hippocampal CA1 Subfield After Transient Global Ischemia

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Delayed neuronal cell death occurs in the vulnerable CA1 subfield of the hippocampus after transient global ischemia (TGI). We demonstrated previously, based on an experimental model of TGI, that the significantly increased content of oxidized proteins in hippocampal CA1 neuron was observed as early as 30 min after TGI, followed by augmentation of PGC-1α expression at 1 hr, as well as up-regulation of mitochondrial uncoupling protein 2 (UCP2) and superoxide dismutases 2 (SOD2). Using the same animal model, the present study investigated the role of calcium/calmodulin-dependent protein kinase IV (CaMKIV) and PGC-1α in delayed neuronal cell death and mitochondrial biogenesis in the hippocampus. In Sprague-Dawley rats, significantly increased expression of nuclear CaMKIV was noted in the hippocampal CA1 subfield as early as 15 min after TGI. In addition, the index of mitochondrial biogenesis, including a mitochondrial DNA-encoded polypeptide, cytochrome c oxidase subunit 1 (COX1), and mitochondrial number significantly increased in the hippocampal CA1 subfield 4 hr after TGI. Application bilaterally into the hippocampal CA1 subfield of an inhibitor of CaMKIV, KN-93, 30 min before TGI attenuated both CaMKIV and PGC-1α expression, followed by down-regulation of UCP2 and SOD2, decrease of COX1 expression and mitochondrial number, heightened protein oxidation, and enhanced hippocampal CA1 neuronal damage. This study provides correlative evidence for the neuroprotective cascade of CaMKIV/PGC-1α which implicates at least in part the mitochondrial antioxidants UCP2 and SOD2 as well as mitochondrial biogenesis in ischemic brain injury. © 2010 Wiley-Liss, Inc.

Key words: calcium/calmodulin-dependent protein kinase IV; peroxisome proliferator-activated receptors γ coactivator-1α; mitochondrial biogenesis; transient global ischemia; hippocampus

Selective neuronal loss in hippocampal CA1 pyramidal neurons is a histological hallmark of transient global ischemia (TGI) and reperfusion, which may occur days after the initial ischemic insult (Pulsinelli et al., 1982; Smith et al., 1984). Despite many theories and
attempts to explain the delayed neuronal death in the CA1 subfield of hippocampus, the underlying mechanisms remain obscure. In addition to its roles as the cellular powerhouse, the mitochondrion is also a key participant in cell death because of its association with an ever-growing list of apoptosis-related proteins (Green and Reed, 1998; Kroemer and Reed, 2000). Emerging evidence suggests that the mitochondrion also plays an important role in delayed neuronal death of the CA1 subfield after TGI (Endo et al., 2006; Miyawaki et al., 2008). Changing cellular metabolic homeostasis and production of reactive oxygen species (ROS) by ischemia may cause the alterations of mitochondrial regulation, turnover, function, content, and biogenesis in the damaged neuronal cells.

Regulation of mitochondrial biogenesis is a complex biological process through the coordinated actions of both nuclear and mitochondrial genomes (Lee and Wei, 2005; Hock and Kralli, 2009). Under the condition of high energy demand, such as endurance exercise training, mitochondrial biogenesis is enhanced in the skeletal muscle cells (Wu et al., 2002; Handschin et al., 2003). Recent evidence also suggests that mitochondrial biogenesis in the neuronal cell may be altered by mitochondrial dysfunction in many pathological settings, including the aging brain, neurodegenerative diseases, and ischemia (Yin et al., 2008; Onyango et al., 2009). However, the signaling mechanism involved in the mitochondrial biogenesis in the ischemic brain damage is still unsettled. Peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) is a transcriptional coactivator that transduces many physiological stimuli into specific metabolic programs, such as gluconeogenesis, thermogenesis, and fatty acid oxidation (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003; Finck and Kelly, 2006). Moreover, PGC-1α has been identified as a major regulator of mitochondrial biogenesis in vivo (Wu et al., 1999). PGC-1α stimulates mitochondrial biogenesis and respiration in muscle cells through an induction of uncoupling protein 2 (UCP2) and regulation of the expression of nuclear respiratory factors (NRFs). NRFs then bind to the promoter of mitochondrial transcription factor A that directly regulates the replication and transcription of mitochondrial DNA (mtDNA; Wu et al., 1999; Puigserver and Spiegelman, 2003). A recent study showed that brain mtDNA content was markedly increased 6 hr after hypoxic/ischemic brain injury in a neonatal rat model (Yin et al., 2008). However, the exact mechanism of PGC-1α underlying the mitochondrial biogenesis in neuronal cells during ischemic injury is not well understood.

Calcium/calmodulin-dependent protein kinase IV (CaMKIV), a nuclear protein kinase, can phosphorylate and activate cAMP response element-binding protein (CREB; Bonni et al., 1999; Riccio et al., 1999; Finkbeiner, 2000) and activate PGC-1α in skeletal muscle cells (Handschin et al., 2003). In transgenic mice, selective expression of a constitutively active CaMKIV may induce expression of PGC-1α and promote mitochondrial biogenesis (Wu et al., 2002). With an experimental model of TGI, we demonstrated previously that, under transient ischemic condition, ROS overproduction may stimulate the activation of PGC-1α and trigger up-regulation of mitochondrial UCP2 in hippocampal CA1 neurons (Chen et al., 2010). Because PGC-1α is a key factor in the signaling cascade of mitochondrial biogenesis, the present study evaluated the hypothesis that CaMKIV may induce PGC-1α activation, which further promotes mitochondrial biogenesis in hippocampal CA1 neurons after TGI. Our results validated this hypothesis by showing that inhibition of CaMKIV may down-regulate the PGC-1α expression, leading to reduced mitochondrial UCP2 expression, and blunt the increase of mitochondrial biogenesis, which further exacerbates oxidative stress and augments neuronal cell death in the hippocampal CA1 subfield after TGI.

MATERIALS AND METHODS

Animals and General Preparations

The experimental procedures used in this study conformed to the guidelines of our institutional committee on experimental animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult male Sprague-Dawley rats (250–325 g) were purchased from the Experimental Animal Center, National Science Council, Taiwan, and housed in an animal room under temperature control (24–25°C) and 12-hr light-dark (08:00–20:00) cycle. Standard laboratory rat chow and tap water were available ad libitum. Animals were anesthetized initially with chloral hydrate (400 mg/kg, i.p.) to perform preparative surgery. The TGI model was used as previously reported (Chen et al., 2006, 2010). Briefly, the animals were subjected to a 10-min period of forebrain global ischemia by clamping both common carotid arteries and lowering blood pressure to 35–40 mmHg by withdrawing blood from a femoral arterial catheter; blood pressure was restored by infusing the withdrawn blood afterward. During anesthesia, the core temperature was monitored and maintained at 37°C ± 0.5°C. The femoral artery was exposed and catheterized with a PE-50 catheter to allow continuous recording of the arterial blood pressure and to keep blood pressure in the designed range. After regaining consciousness, the animals were maintained in an air-conditioned room at 25°C.

Pharmacological Pretreatments

In those experiments involving pharmacological pretreatments, test agents were microinjected bilaterally and sequentially into the CA1 subfield of hippocampus, at a volume of 100 nl on each side. Drug delivery into the hippocampal CA1 subfield was carried out with a stereotaxically positioned 27-gauge stainless-steel needle connected to a 0.5-μl Hamilton microsyringe (Hamilton, Reno, NV; Chen et al., 2006, 2010; Chuang et al., 2009). As shown recently (Chen et al., 2006, 2010), the stereotaxic coordinates used for the hippocampal CA1 subfield were based on the rat brain atlas ( Paxinos and Watson, 2007): 3.2–3.4 mm posterior to bregma, 1.8–2.0 mm from the midline, and 3.4–3.6 mm below the
cortical surface. A CaMK IV inhibitor, KN-93 (Sigma-Aldrich, St. Louis, MO; Sato et al., 2006; Zhang et al., 2008), 10 μmol in 100 nl artificial cerebrospinal fluid (aCSF), was given 30 min before ischemia. Rats that had received bilateral microinjection of the same amount of aCSF served as vehicle controls. The composition of aCSF was (mM): NaCl 117, NaHCO3 25, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaH2PO4 1.2, and glucose 11, pH 7.3–7.4. Animals that had received choral hydrate anesthesia and surgical preparations without additional experimental manipulations served as sham-controls.

Detection of Protein Oxidation

Oxidized protein was detected by using a protein oxidation detection kit (OxyBlot; Chemicon, Temecula, CA). This kit provides reagents for sensitive immunodetection of carbonyl group, which is a hallmark of the oxidation status of proteins (Singhal et al., 2002; Chen et al., 2010). Protein extracts from the hippocampal CA1 subfield at various time points after ischemia and reperfusion were reacted with 2,4-dinitrophenylhydrazine and derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone; Smith et al., 1991). The DNP-derivatized protein samples were separated on a 15% SDS-polyacrylamide gel, followed by Western blotting. The blot was incubated with a primary antibody with rabbit anti-DNP antibody, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody according to the manufacturer’s instructions.

Collection of Tissue Samples From the Hippocampus

At predetermined time intervals (15 or 30 min; 1, 4, 24, or 48 hR; or 4 days) after induction of TGI, rats were again anesthetized with choral hydrate (400 mg/kg, i.p.) and perfused intracardially with 50 ml of warm (37°C) saline containing heparin (100 U/ml). The brain was rapidly removed under visual inspection and placed on a piece of gauze moistened with ice-cold 0.9% saline. We routinely collected tissues from bilateral hippocampal CA1 area, and these samples were stored at −80°C until biochemical analyses (Chen et al., 2006, 2010). The concentration of total proteins extracted from tissue samples was determined by the BCA Protein Assay (Pierce, Rockford, IL). In selected experiments, proteins from the nuclear fraction of the hippocampal samples were extracted by a commercial kit (Active Motif, Carlsbad, CA).

Western Blot Analysis

Western blot analysis for CaMKIV, PGC-1α, UCP2, SOD2, and cytochrome c oxidase subunit I (COX1) was carried out on proteins extracted from total lysate or nuclear fractions of hippocampal samples (Chen et al., 2006, 2010; Chuang et al., 2009). The primary antisera used included a rabbit polyclonal antiserum against PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA) or SOD2 (Santa Cruz Biotechnology); a goat polyclonal antiserum against UCP2 (Santa Cruz Biotechnology); or a mouse monoclonal antiserum against CaMKIV (Santa Cruz Biotechnology), COX1 (Abcam, Cambridge, United Kingdom), or α-tubulin (Santa Cruz Biotechnology). The secondary antiserum used included a horseradish peroxidase-conjugated goat anti-rabbit (Chemicon) for PGC-1α or SOD2, a donkey anti-goat (Chemicon) for UCP-2, and a goat anti-mouse IgG (Chemicon) for CaMKIV, COX1, or α-tubulin. Specific antibody–antigen complex was detected by an enhanced chemiluminescence Western blot detection system (NEL, Boston, MA). The amount of protein was quantified in ImageMaster software (Amersham Pharmacia Biotech, Piscataway, NJ) and was expressed as the ratio relative to α-tubulin.

Electron Microscopic Evaluation of Mitochondrial Number

At 4 hr after TGI, bilateral CA1 subfields of hippocampus were removed and processed for electron microscopy (Chuang et al., 2004; Chang et al., 2009). Tissue samples were diced and submerged in 4% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.2). Tissues were postfixed with osmium and stained en bloc with uranyl acetate. After dehydration, each specimen was embedded by infiltration in Spurr’s medium. After trimming of the tissue blocks, sections were cut to a thickness of 90 nm, poststained with uranyl acetate and lead citrate, and viewed on 300-mesh-coated grids using a JEOL JEM-1230 (Tokyo, Japan) electron microscope. For measurement of mitochondrial number, 15 randomly selected CA1 areas per animal, which included large neuronal-like nuclei covering about one-fourth of the visible image, were photographed at ×8,000 magnification and counted according to the method described by Yin et al. (2008), with minor modifications.

Quantitative Analysis of DNA Fragmentation

Tissue sampling from hippocampal CA1 subfield subjected to quantitative analysis of DNA fragmentation was reported previously (Chuang et al., 2007, 2009; Chen et al., 2010). To quantify apoptosis-related DNA fragmentation, a cell death enzyme–linked immunosorbent assay (catalog No. 11774425001; Roche Molecular Biochemicals, Mannheim, Germany) was used to assay the level of histone-associated DNA fragments in the cytoplasm (Okuno et al., 2004; Chuang et al., 2007, 2009; Chen et al., 2010). The amount of nucleosomes in the cytoplasm was quantitatively determined by using 2,2′-azino-di-[3-ethylbenzthiazoline] sulfonate as the substrate. Absorbance was measured at 405 nm and referenced at 490 nm with a microtiter plate reader (Anthros Labtec, Salzburg, Austria).

Quantitative Assessment of Neuronal Loss in the Hippocampal CA1 Subfield

Animals were processed for histopathological analysis of the severity of neuronal cell loss in the hippocampus 4 days after the TGI. For this purpose, 10-μm paraffin–embedded brain sections were deparaffinized in two exchanges of xylene for 5 min each; washed sequentially in 100%, 95%, and 70% ethanol; and stained with cresyl violet (Chen et al., 2006, 2010; Chuang et al., 2009). Pyramidal neurons were counted in a double-blind manner in the middle CA1 region of the hippocampus. Twenty (250-μm)² fields, five regularly chosen sections about 200 μm apart in each section, four fields per section, on treatment with sham control, KN-93, or vehicle control were counted from each brain sample. The numbers of surviving neurons are denoted as “surviving neurons/(250 μm)².”
Statistical Analysis

All values are expressed as mean ± SEM. One-way ANOVA was used, as appropriate, to assess group means, followed by the Scheffe multiple-range test for post hoc assessment of individual means. P < 0.05 indicates statistical significance.

RESULTS

Temporal Changes of CaMKIV Expression in the Hippocampal CA1 Subfield After TGI

Our first series of experiments established an increase of CaMKIV expression in the hippocampal CA1 subfield after TGI. Western blot analysis (Fig. 1) revealed that CaMKIV expression in the nuclear protein extracted from the hippocampal CA1 subfield underwent a significant increase as early as 15 min that peaked at 30 min after TGI. Moreover, the augmented expression of CaMKIV lasted for more than 48 hr after TGI (Fig. 1).

CaMKIV Regulates PGC-1α Expression in the Hippocampal CA1 Subfield After TGI

We reported recently (Chen et al., 2010) a significant increase of PGC-1α expression in the hippocampal CA1 subfield that peaked at 4 hr after TGI. Because PGC-1α plays a pivotal role in ischemic brain injury and CaMKIV may regulate the expression of PGC-1α (Wu et al., 2002), our second series of experiments established that activation of CaMKIV underlies this up-regulation of PGC-1α expression. Compared with aCSF-pretreated animals, microinjection bilaterally into the CA1 subfield of a CaMKIV inhibitor, KN-93 (10 μmol), significantly retarded the augmentation of CaMKIV expression (Fig. 2A) in the CA1 subfields detected 30 min and PGC-1α expression (Fig. 2B) detected 4 hr after TGI.

Effects of KN-93 on COX1 Expression and Mitochondrial Number in the Hippocampal CA1 Subfield After TGI

PGC-1α is a transcriptional coactivator that transduces many physiological stimuli into specific metabolic programs, including mitochondrial biogenesis (Finck and Kelly, 2006). During the early stage of TGI in rats, we demonstrated that up-regulation of PGC-1α expression occurred in hippocampal CA1 neurons (Chen et al., 2010). Because the activation of PGC-1α may promote mitochondrial biogenesis (Wu et al., 1999), our third series of experiments examined whether mitochondrial biogenesis in the hippocampus changes following TGI. Western blot analysis revealed a significant increase of the mitochondrial DNA-encoded polypeptide COX1 in the hippocampal CA1 subfield as early as 1 hr that peaked at 4 hr after TGI (Fig. 3). With the observation that the surge in COX1 at the CA1 subfield of hippocampus exhibited a time course that was compatible with the up-regulation of PGC-1α expression after TGI (Chen et al., 2010), it is of interest to evaluate the relationship between CaMKIV/PGC-1α signaling pathway and mitochondrial biogenesis. Compared with aCSF-pretreated animals, microinjection bilaterally into the CA1 subfield of KN-93 (10 μmol) significantly decreased the augmentation of PGC-1α (Fig. 2B) and COX1 expression (Fig. 4A) in the CA1 subfields detected 4 hr after TGI. To confirm that the changes in COX1 level demonstrated in our biochemical analyses on protein extracts from hippocampal CA1 subfield indeed resulted from mitochondrial biogenesis, we examined and quantified the mitochondrial number in hippocampal CA1 neurons (Fig. 4B,C). Electron microscopy showed a significant increase of mitochondrial number in hippocampal CA1 pyramidal neurons 4 hr after TGI (Fig. 4B,C). Inhibition of CaMKIV by pretreatment with KN-93 (10 μmol) retarded the increment of mitochondrial number in the CA1 neurons (Fig. 4B,C).

Effects of KN-93 on UCP2 and SOD2 Expressions in the Hippocampal CA1 Subfield After TGI

We recently reported that a temporal correlation existed between the increased PGC-1α expression and up-regulated UCP2 and SOD2 in the hippocampal CA1 subfield after TGI (Chen et al., 2010). Therefore, our
forth series of experiments investigated whether the CaMKIV/PGC-1α signaling pathway causally regulates UCP2 and SOD2 expressions under ischemic condition. Compared with animals pretreated with aCSF, microinjection into the bilateral CA1 subfield of KN-93 (10 μmol) significantly prevented the augmentation of UCP2 and SOD2 expressions in the hippocampal CA1 subfield (Fig. 5) detected 24 hr after induction of TGI.

Effect of KN-93 on Oxidative Stress and Neuronal Cell Death in the Hippocampal CA1 Subfield After TGI

Our group reported previously (Chen et al., 2006, 2010) that an excessive production of ROS underlies neuronal cell death in the CA1 subfield of hippocampus after TGI. Knock-down of PGC-1α by antisense strategy also down-regulated the expression of UCP2 and SOD2, together promoting oxidative stress and neuronal cell death in the CA1 subfield of hippocampus after TGI (Chen et al., 2010). Our final series of experiments examined whether the TGI-induced oxidative stress and neuronal injuries in the hippocampal CA1 subfield are linked to the CaMKIV/PGC-1α signaling pathway. We found that the extent of protein oxidation in the hippocampal CA1 subfield 24 hr after TGI was significantly increased by pretreatment with KN-93 (10 μmol; Fig.

Fig. 2. CaMKIV regulates PGC-1α expression in hippocampal CA1 subfield after TGI. Rats were microinjected into bilateral CA1 subfields with aCSF or KN-93 (10 μmol) 30 min before TGI. Nuclear proteins were isolated from collected hippocampal CA1 subfield from sham-operated controls or animals 30 min after 10 min of TGI for Western analysis of CaMKIV (A) or 4 hr after 10 min of TGI for Western analysis of PGC-1α (B). The same blots were also probed with α-tubulin antibody to serve as internal reference control for equal loading of proteins in each lane. Values are mean ± SEM from four to six animals per experimental group. *P < 0.05, †P < 0.005 vs. sham-control group; ‡P < 0.05 vs. aCSF + TGI group in the Scheffé multiple-range test.

Fig. 3. Temporal changes of COX1 expressions in the hippocampal CA1 subfield after TGI. Hippocampal CA1 samples were collected from the rats at indicated times after 10 min of TGI or from sham-operated controls (S), followed by total protein extraction and Western analyses for detection of COX1. The same blots were also probed with α-tubulin antibody to serve as internal reference control for equal loading of proteins in each lane. Representative blots (inset) and quantitative analyses from four to six animals per experimental group are shown. Values are mean ± SEM. *P < 0.05, †P < 0.005, ‡P < 0.001 vs. sham-control group in the Scheffé multiple-range test.
6). Quantitative analysis of histone-associated DNA fragments in the cytoplasm (Fig. 7A) revealed that cell death 48 hr after the induction of TGI was significantly exacerbated by pretreatment with microinjection into the hippocampal CA1 field of KN-93 (10 μmol). Quantitative assessment of neuronal cell death also showed that the characteristic delayed neuronal cell loss in the CA1 subfield 4 days after TGI was significantly increased by pretreatment with KN-93 (10 μmol) in the hippocampus (Fig. 7B).

Histology

Based on the location of the tip of the microinjection needle, histological verifications indicated that our results were obtained from animals that received local application of KN-93 or aCSF to the CA1 subfield of hippocampus. Microinjection of KN-93 to sites adjacent to the CA1 subfield was ineffective.

DISCUSSION

Based on an experimental model of TGI, the present study provides novel evidence that the CaMKIV/PGC-1α signaling pathway may regulate the expression of mitochondrial ROS-detoxifying enzymes, namely, UCP 2 and SOD 2, as well as mitochondrial biogenesis, thereby providing an endogenous protective effect against delayed neuronal cell death induced by ischemia in the vulnerable hippocampal CA1 area. By using a CaMKIV inhibitor, KN-93, we found that inhibition of CaMKIV activity may reduce the expression of PGC-1α and further attenuate the ischemia-induced augmentation of mitochondrial UCP2 and SOD2 expression, mitochondrial content, and mitochondrial numbers, leading

Fig. 4. Effects of KN-93 on COX1 expression and mitochondrial number in the hippocampal CA1 subfield after TGI. Rats were microinjected into bilateral CA1 subfields with aCSF or KN-93 (10 μmol) 30 min before TGI. Hippocampal CA1 samples were collected 4 hr after TGI or from sham-operated controls, followed by total protein extraction and Western analyses for detection of COX1 (A). The same blots were also probed with α-tubulin antibody to serve as internal reference control for equal loading of proteins in each lane. Representative blots (inset) and quantitative analyses from four to six animals per experimental group are shown. Representative electron photomicrographs (B) show ultrastructure of pyramidal cells containing the nucleus (Nu) and mitochondria (arrows) in the hippocampal CA1 subfield from the rats microinjected into bilateral CA1 subfields with aCSF or KN-93 (10 μmol) 4 hr after 10 min of TGI or from sham-operated controls. C: Quantitative analysis of the mitochondrial number in the electron photomicrographs from three animals per experimental group. Values in A,C are mean ± SEM. *P < 0.05, †P < 0.005 vs. sham-control group; ‡P < 0.05 vs. aCSF + TGI group in the Scheffee multiple-range test. Scale bar = 1 μm.
to enhancement of oxidative stress and increased neuronal cell death in the hippocampal CA1 subfield after TGI. These results may indicate the importance of the CaMKIV/PGC-1α signaling cascade in delayed neuronal damage in the hippocampal CA1 subfield after TGI.

Reactive oxygen radicals have been implicated in brain injury after cerebral ischemia and reperfusion (Chan, 2001). ROS are also a mediator in signaling involving mitochondria, DNA repair enzymes, and transcription factors that may regulate cellular survival or death in vulnerable hippocampal CA1 neurons after cerebral ischemia (Chan, 2001; Chen et al., 2001, 2010;
In brain cells, PGC-1α plays a key role in regulating the basal level of the ROS defense system and is involved in the induction of the ROS-detoxifying enzymes upon oxidative stress challenge (Handschin and Spiegelman, 2006; St.-Pierre et al., 2006; Chen et al., 2010).

Work in our laboratory demonstrated recently that, under transient ischemic conditions, ROS overproduction may stimulate the activation of PGC-1α signaling pathway, further triggering up-regulation of UCP2 and SOD2 in hippocampal CA1 neurons. Down-regulation of PGC-1α expression diminished the expression of UCP2 and SOD2 and augmented oxidative stress and neuronal cell death (Chen et al., 2010). UCP2 and SOD2 are two mitochondrial proteins crucial for neuronal survival after ischemic stroke (Chan, 2001; Mattiasson et al., 2003). Up-regulation of UCP2 after cerebral ischemia decreased the release of ROS and reduced neuronal loss in the hippocampus that offers a neuroprotection against ischemic brain injury (Mattiasson et al., 2003; Chen et al., 2006; Deierborg et al., 2008). Furthermore, animals that overexpress SOD2 showed heightened resistance against oxidative stress-induced neuronal injury after transient focal cerebral ischemia (Keller et al., 1998; Noshita et al., 2001). We noted that a quick activation of nuclear CaMKIV began as early as 15 min and peaked at 30 min after TGI. Pretreatment with KN-93, an inhibitor of CaMKIV, in the hippocampus significantly blunted the surge in PGC-1α expression and activation of UCP2 and SOD2 in the CA1 neurons induced by experimental TGI. These results support the notion that CaMKIV is upstream to PGC-1α signaling pathway for regulation of the mitochondrial antioxidant defense system and ROS metabolism in hippocampal CA1 neurons after ischemia.

PGC-1α has been shown to induce mitochondrial biogenesis and greatly increase respiration in liver, fat, skeletal, or cardiac muscle cells (Wu et al., 1999; Lehman et al., 2000; Handschin et al., 2003; Puigserver and Spiegelman, 2003). In muscle cells, PGC-1α gene expression is regulated by calcium-signaling components, such as CaMKIV and the protein phosphatase calcineurin A (Wu et al., 2002; Handschin et al., 2003). Emerging evidence demonstrated that CaMKIV regulated PGC-1α expression and augmented mitochondrial DNA replication and mitochondrial biogenesis in muscle cells (Wu et al., 1999; Handschin et al., 2003). CaMKIV likely activates PGC-1α through the binding of CREB to the PGC-1α promoter (Handschin et al., 2003). However, in neuronal cells, the mechanisms underlying CaMKIV/PGC-1α signaling pathway in mitochondrial biogenesis are not well understood, particularly during ischemic injury. CREB phosphorylation in neurons after ischemia and exposure to glutamate may subsequently activate CaMK II–IV and provide a neuroprotective response (Mabuchi et al., 2001; Blanquet et al., 2006). A recent report revealed that nuclear CaMKIV requires constitutively active CREB to promote spiral ganglion neurons survival in neonatal rats (Bok et al., 2007). Moreover, evidence suggests that CaMKIV, CREB, and brain-derived neurotrophic factor might be mediators of neuronal survival and plasticity in the suprapyramidal subfield of the dentate gyrus under ischemic conditions.
ischemia, the activation of PGC-1α expression, followed by the decrease of UCP2 and SOD2 expression and increase of oxidative stress that augments the neuronal cell death in the hippocampus after TGI. We suggest, based on the cascade of cellular events after pretreatment with KN-93, that the activation of CaMKIV under ischemic conditions may provide an endogenous protective effect against ischemia-induced neuronal cell death in the hippocampal CA1 subfield.

The mitochondrion is an important organelle for cellular homeostasis. It plays essential roles in regulation of energy metabolism, generation of ROS, and regulation of cell death in response to different brain injuries, including ischemic damage. Recent evidence demonstrated that mitochondrial biogenesis was rapidly increased 6–24 hr after hypoxic-ischemic brain injury, and the increase in mitochondrial biogenesis was suggested to be a novel component of the endogenous repair mechanisms of the brain (Yin et al., 2008). After hypoxic-ischemia, mtDNA content, total mitochondrial number, expression of the mitochondrial transcription factors downstream of PGC-1α and the mitochondrial proteins increased in cortical neurons of rats (Yin et al., 2008). PGC-1α can stimulate the expression of NRF2 and mitochondrial transcription factor A to promote mitochondrial biogenesis by activating expression of nuclear and mitochondrial genes encoding mitochondrial proteins (Wu et al., 1999; Puigserver and Spiegelman, 2003; Finck and Kelly, 2006). Our recent study (Chen et al., 2010) demonstrated that, under transient ischemic conditions, PGC-1α expression may activate and trigger up-regulation of mitochondrial UCP2 in hippocampal CA1 neurons. In the present study, we found mtDNA encoding polypeptide COX1 and total mitochondrial number were increased in hippocampal CA1 neurons 4 hr after TGI. Down-regulation of CaMKIV resulted in diminishing PGC-1α expression and further decreased COX1 expression and mitochondrial number in the hippocampal CA1 neurons. PGC-1α also stimulates mitochondrial biogenesis through induction of UCP2 (Wu et al., 1999), which suggests that the increase of mitochondrial biogenesis in hippocampal CA1 neurons in response to transient ischemic conditions may be related to the activation of PGC-1α/UCP2 signaling pathway. However, the causal relationship between the number and function of the mitochondrion requires further delineation.

We are cognizant that the role of PGC-1α in mitochondrial biogenesis of neurons, however, remains controversial. The recent report (Yin et al., 2008) revealed an increased mitochondrial biogenesis after focal hypoxic-ischemic brain injury without a significant change in PGC-1α expression in cortical neurons. Nevertheless, transient hypoxic-ischemic preconditioning stimulates mitochondrial biogenesis in subcortex of mice by a neuronal nitric oxide synthase-dependent up-regulation of PGC-1α expression (Gutsaeva et al., 2008). In the present study, we also established that, after transient ischemia, the activation of PGC-1α expression may contribute to the increased mitochondrial biogenesis in hippocampal CA1 neurons. The discrepancy between PGC-1α expression and mitochondrial biogenesis may relate to differences in animal models, brain tissues to be studied, and experimental methods. Further investigations on these issues are warranted.

We are aware that the conflict results exist among the effects of KN-93 in mediating cell death under different pathological conditions. KN-93 may also inhibit the CaMKII signaling pathway that provides the protective effects in cardiac cell injury during ischemia-reperfusion (Salas et al., 2010) and neuronal cell death induced by depolarization (Takano et al., 2003). Otherwise, pretreatment with KN93 (30 μM) 30 min before exposure to glutamate significantly increased the level of neuronal damage compared with controls, suggesting that CaMK-mediated CREB phosphorylation may contribute to neuronal survival by inhibition of both CaMKII and -IV (Mabuchi et al., 2001). However, CaMKII and -IV mediate distinct prosurvival signaling pathways in response to depolarization in neurons (Bok et al., 2007). CaMKIV, but not CaMKII, requires CREB and activates the PGC-1α signaling pathway to promote neuron survival. With a pharmacological approach, KN-93 is accepted as a CaMKIV inhibitor (Sato et al., 2006; Zhang et al., 2008). The selectivity of KN-93 may affect the interpretation of our results. In this regard, our results from pharmacological blockade of KN-93 in the hippocampal CA1 were corroborated by complementary inhibition of in CaMKIV and PGC-1α protein expression induced by TGI in the hippocampal CA1 subfield. Thus, these results indicate that KN-93 may inhibit the CaMKIV/CREB and PGC-1α signaling pathway and contribute to the deleterious effect in hippocampal CA1 neurons after TGI. Moreover, differences in animal models and experimental design may account for the discrepancies between our observations and the reported findings.

In conclusion, our study provides novel evidence to demonstrate that, under transient ischemic conditions, the CaMKIV/PGC-1α signaling pathway is activated, which may trigger the UCP2 and SOD2 expression and promote mitochondrial biogenesis in the hippocampal CA1 subfield. In keeping with the role of mitochondria biogenesis as a potential endogenous protective mechanism (Yin et al., 2008; Vosler et al., 2009), boosting the signal transduction pathways upstream of mitochondrial biogenesis, such as the CaMKIV/PGC-1α signaling cascade, may therefore become a novel target for a therapeutic strategy against ischemic brain damage.

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