CPG15, a New Factor Upregulated after Ischemic Brain Injury, Contributes to Neuronal Network Re-Establishment after Glutamate-Induced Injury

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ABSTRACT

Candidate plasticity-related gene 15 (cpg15) encodes a protein that regulates dendritic and axonal arbor growth and synaptic maturation. In the present study, we investigated the potential role of CPG15 in regulating the neuronal network re-establishment after ischemic brain injury. In the mouse model with transient global ischemia (TGI), CPG15 transcripts and proteins were determined using RT-PCR and Western blot analyses. Cell proliferation was observed using 5'-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) labeling. Double immunostaining and depletion of soluble CPG15 proteins were performed to examine the cellular distribution of CPG15 and the role of soluble CPG15 in the neurite outgrowth during the neuronal network re-establishment in primarily cultured hippocampal cells after glutamate-induced injury. We demonstrated that CPG15 expression in the hippocampus was upregulated at 1–2 weeks after TGI. In the dentate gyrus, the number of CPG15 and BrdU positive cells increased concurrently after the injury. During the neuronal network re-establishment after the glutamate-induced injury of primarily cultured hippocampal cells, CPG15 was mainly located at the ends and turn-off regions of the growth cones and in the vesicles. Depletion of soluble CPG15 proteins secreted from the hippocampal cells in the culture media significantly reduced the neurite outgrowth and neuron-neuron connection. The results indicate that CPG15 may function as a new factor required in re-establishment of neuronal network after the injury. Our findings will be important in developing a new strategy to enhance endogenous neurogenesis after an ischemic brain injury.

Key words: CPG15; mice; neuronal network; transient global ischemia

INTRODUCTION

Each year, millions of people in the world suffer a stroke that causes incapacitating brain injury and incurs a huge amount of health-care expense. Recent advances in the acute treatment of strokes have improved survival rates. However, the long-term morbidity after stroke remains unacceptably high. Progress in replacing dead neurons with new ones through transplantation of stem cells or stimulation of neurogenesis from endogenous neural progenitors offers the exciting possibility of addressing this devastating problem by partially recon-
soluble CPG15 proteins are required in the re-establishment of neuronal network after the injury. In addition, CPG15 is upregulated after the ischemic brain injury or the injury, such as after an ischemic brain injury. This factor in re-establishment of the neuronal network after ischemia remain to be identified.

Candidate plasticity-related gene 15 (cpg15), encoding a protein (CPG15) with a calculated molecular mass of 15 kDa, was first identified in the hippocampus as a gene induced by neural activity (Nedivi et al., 1993, 1996). CPG15 contains a secretory signal peptide composed of 27 amino acid residues at the N terminus and a cleavage signal peptide (27–amino acid residues) of glycosyl-phosphatidylinositol (GPI)-anchored proteins at the C terminus and functions as secreted and/or extracellular GPI-anchored membrane proteins (Nedivi et al., 1996). CPG15 expression is correlated with afferent nerve growth, dendritic elaboration and synaptogenesis (Naeve et al., 1997; Cantllops et al., 2000; Javaherian and Cline, 2005). Studies indicate that cpg15 functions as an immediate-early gene (IEG) or a target of synaptic plasticity-related signal transduction pathways (Fujino et al., 2003). Several studies demonstrate that cpg15 expression is induced by glutamate receptor agonists in the brain, in the developmental visual cortex by light, in post-mitotic neurons after seizures and in neuronal plasticity during development or after spinal cord injury (Nedivi et al., 1993, 1998; Naeve et al., 1997; Lee and Nedivi, 2002; Corriveau et al., 1999; Elliott et al., 2003). Recent studies reported that the soluble CPG15 protects cortical progenitors from apoptosis by inhibiting caspase 3 activities (Di Giovanni et al., 2005). These studies suggest that CPG15 may play important roles in functional recovery after the brain injury or in the apoptosis in early brain development. Therefore, we hypothesize that in addition to its function as an IEG, CPG15 may act as a later factor in re-establishment of the neuronal network after the injury, such as after an ischemic brain injury. This hypothesis is supported by the present study showing that CPG15 is upregulated after the ischemic brain injury or glutamate-induced hippocampal cell injury. In addition, soluble CPG15 proteins are required in the re-establishment of neuronal network after the injury. The results by testing the hypothesis add new knowledge regarding the potential role of CPG15 as a promoter of neurite outgrowth in improving the recovery after a stroke.

METHODS

Transient Global Ischemia in Mice

The use of adult male mice (27–37 g) was approved by the Natural Science Foundation of China and ICACU at the University of Utah. The animals were anesthetized with 2% chloral hydrate. After median neck incisions, both common carotid arteries (CCAs) were exposed and occluded by tying the nylon thread separately around both CCAs for exactly 15 min. The knot was then removed to restore the cerebral blood flow. Sham-operated animals were treated identically, except that the CCAs were not occluded after the neck incisions.

Immunohistochemistry

For IHC, at 7, 14, and 21 days (7d, 14d, and 21d) after TGI, the animals were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused with saline followed by 4% paraformaldehyde. For 5′-bromo-2′-deoxyuridine-5′-monophosphate (BrdU; Sigma) labeling, at 7d, 14d, and 21d after TGI, animals were injected intraperitoneally with BrdU (50 mg/kg; Sigma) followed by two consecutive injections at 8-h intervals before killed (Zhu et al., 2003). After perfusion, serial sections (20 μm) of the brains were cut to encompass the entire hippocampus using a freezing microtome. For immunocytochemistry (ICC), the cultured cells were fixed with 4% paraformaldehyde. The sections or fixed cells were washed in PBS, incubated in 1% H2O2 in PBS for 30 min to block endogenous peroxidases and then for 1 h in 5% normal horse serum, 0.5% Triton X-100, and 0.2% BSA in PBS. The sections were then incubated with goat anti-CPG15 (1:500; R&D) or mouse anti-BrdU antibodies (1:500; Sigma) overnight at 4°C. HRP-conjugated horse anti-goat IgG or anti-mouse antibodies (1:500; Sigma) were used as secondary antibodies. Control sections were processed with omission of the primary antibodies. Signals were developed by DAB substrates. The CPG15 or BrdU-positive cell numbers per section were calculated by averaging the total cell numbers of 20 brain sections (every third section of the serial sections through the hippocampus) within the same ischemic area of subgranular zone (SGZ) where the neurogenesis was triggered by ischemia. Adjacent sections were used to analyze CPG15 or BrdU positive cells. Total six independent experiments were performed.

Apoptotic cells were analyzed using a TUNEL kit (Oncogene) following the supplier’s protocol. Double
immunostaining of CPG15 and markers (VAMP-2 for vesicles, 1:500, Calbiochem; TUC-4 for growth-cones, 1:500, Chemicon) were performed with fluorescence and confocal microscope (Zeiss).

**Cell Cultures and CPG15 Antibody Absorption**

Primarily hippocampal cell culture was performed as described previously (Brewer et al., 1993). In brief, after protease digestion of mouse embryonic (E14) hippocampal tissues at 37°C for 15 min, the cells were dissociated with a fire-polished Pasteur pipette in minimal essential medium containing Earle’s salts, 10% fetal bovine serum, 0.5% glucose, 1 mmol/L sodium pyruvate, 25 μmol/L glutamine, and 1 × penicillin/streptomycin. Cells were then plated onto glass cover-slips coated with poly-L-lysine and laminin at a density around 100–200 cells/mm² and maintained in the neurobasal culture medium (Invitrogen) containing 1/100 B27 supplement and 1/100 penicillin/streptomycin in an incubator with 5% CO₂ at 37°C. For glutamate treatment, the cells were cultured in the media containing added glutamate (0.5 g/L) for 24 h.

For CPG15 antibody absorption (24 h), after culture for 48 h, mouse CPG15 antibodies (25 μg/mL) were added into the medium. The total unconnected neurite numbers under 10 randomly selected microscopic fields for each culture plate (total three culture plates) were counted for calculating the average unconnected neurite numbers per microscopic field. To calculate the neurite numbers per dendrite, total 20 dendrites with more than one neurite for each dendrite were randomly used to count the average neurite numbers for each dendrite.

**Reverse Transcription–Polymerase Chain Reaction, Western Blotting, and Enzyme-Linked Immunosorbent Assay**

Total RNA was extracted from the hippocampus or cultured cells using Trizol reagent (Sigma) following the supplier’s instructions. RT reactions were performed as described previously (Xu et al., 2004). The primer sequences for PCR amplification were 5’-ATG GGA CTT AAG TTG AAC GG-3’ (CPG15, forward), 5’-TCA GAA GAG CCA GGT CG-3’ (CPG15, reverse), 5’-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3’ (β-actin, forward) and 5’-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3’ (β-actin, reverse). PCR conditions were denaturing at 94°C for 2 min followed by 30 cycles (25 cycles for β-actin) of denaturing at 94°C for 20 sec, annealing at 56°C for 20 sec and extension at 72°C for 30 sec, and ended by 5 min extension at 72°C. PCR products were confirmed by sequencing analysis. The CPG15 transcript levels were semiquantified by determining the ratio of CPG15 to β-actin band intensities following 1% agarose gel electrophoresis after EthBr staining. The image analysis was performed using Furi Image Instrument with Multi-Analyst software (Furi, Shanghai).

Western blotting was performed as described previously (Chen et al., 2005) except that goat anti-CPG15 (1:200) or rabbit anti-β-actin (1:500, Sigma) antibodies were used as primary antibodies.

For competitive ELISA, the diluted recombinant CPG15 (40 μL/mL) in PBS solution were added to a 96-well plate (50 μL/well) and then incubated overnight at 4°C. After three washes, the plates were incubated in blocking solution for 30 min. The medium from the cell culture was di-

**FIG. 1.** The CPG15 expression is upregulated after TGI. (I) TUNEL-labeled cells in CA1, and CA3 at 1 day after TGI. (a) Ischemic CA1 region. (b) Control CA1 region. (Insets, top right corners) Magnified views of the area in the black frame. (c) Ischemic CA3 region. (d) Control CA3 region. The arrows indicate the positive cells with TUNEL staining. Scale bar = 100 μm. (II) CPG15 expression in the hippocampus after TGI. Gel images show the CPG15 mRNA (a) or protein (b) levels at various days after TGI. (c,d) The statistical results of CPG15 mRNA or protein levels.
luted (40 µL/300 µL) in the blocking solution containing 0.8 µg/mL mouse CPG15 antibodies. The diluted medium was then added to the wells to replace the blocking solution followed by incubation for 2 h. After three washes, the plates were incubated for 2 h with AP-conjugated horse anti-mouse IgG antibodies (Vector). After washing, the bound immunocomplexes were detected with pNPP-Na (3 mmol/mL) in boric acid buffer solution (pH 9.8). The optical density (OD) was measured at 405 nm.

Statistical Analysis

Experiments were repeated at least three times independently in triplicates. Data are presented as mean ± SD. The difference was determined as significant by Student’s t-test at \( p < 0.05 \) or most significant at \( p < 0.01 \), as indicated by one or two asterisks in the figures, respectively.

RESULTS

Transient Upregulation of CPG15 Expression after Mouse TGI

To begin to examine the potential role of CPG15 in re-establishment of the neuronal network after the ischemic brain injury, we characterized the temporal profile of CPG15 expression in mouse hippocampus after TGI. In the study, we used the TUNEL analysis of apoptotic cells to determine the ischemic hippocampal injury (Xu et al., 1996). Figures 1-Ia and -Ic demonstrated that TGI elicited apoptosis in the CA1 and CA3 regions, compared to the control (Figs. 1-Ib and Id). The expression of CPG15 transcripts and proteins in the hippocampus was determined using RT-PCR (Figs. 1-IIa and -IIC) and Western blot (Figs. 1-IIb and -IID) analyses with β-actin as an internal control. The results showed that CPG15 expression was significantly upregulated between 1 and 2 weeks after TGI compared to that in sham-operated animals. The expression reached a peak increase at 14d after TGI (Figs. 1-IIc and -IID), an approximately 4–5-fold increase in either mRNA or protein levels, suggesting that CPG15 may be functionally required during 1–2 weeks after the ischemic brain injury.

Concurrent Increases in the Number of CPG15- and BrdU-Positive Cells in the Dentate Gyrus and Expression of CPG15 in CA1 Region after TGI

Neural cell proliferation is a critical process in neurogenesis after the ischemic brain injury (Liu et al., 1998). To examine the possibility that the upregulated CPG15 expression may be related to the neurogenesis, we immunohistochemically examined the time-course of cell proliferation (labeled with BrdU) (Liu et al., 1998) and CPG15 expression in the DG after TGI. Representative images in Figures 2-Ia–f showed that BrdU- and CPG15-positive cell numbers increased in the DG between 1 and 2 weeks after the injury. Figures 2-Ig and 2-Ih provide a quantitative assessment of BrdU- and CPG15-positive cell numbers per section. The results demonstrated that the increase in BrdU-positive cell numbers after the injury had a temporal pattern parallel to that in CPG15 (with a concurrent correlation coefficient \( r = 0.878 \), data

FIG. 2. After TGI-, BrdU-, and CPG15-positive cells in the DG were increased concurrently and the CPG15 expression along the neurites in CA1 region was increased. (I) Temporal profiles of BrdU- and CPG15-positive cells in the DG after TGI. The BrdU-positive (a–e) or CPG15-positive (d–f) cells in the DG at 7, 14, and 21 days after TGI. Scale bar = 100 µm. The arrow indicates the BrdU-positive cells. (g,h) Statistic analyses of BrdU- or CPG15-positive cell numbers, respectively. (II) IHC images showing the enhanced expression of CPG15 along the neurites in CA1 region 14 days after ischemia (k,l) compared with the sham-operated animals (i,j). Scale bar = 20 µm. j and l are magnified images of i and k, respectively. The arrow indicates the CPG15 expression in one of the neurites.
not shown). At 14d, the population of both BrdU- and CPG15-positive cells reached a peak. The concurrent increase in CPG15- and BrdU-positive cell numbers suggests that there is a potential relationship between the upregulated CPG15 expression and cell proliferation in the DG during the recovery after the injury. In addition, Figure 2-II showed that CPG15 expression was enhanced along the neurites in the dendritic zone of CA1 region at 14d after TGI (k and l) compared with the sham-operated animals (i and j), indicating that the increased CPG15 may occur in new cells migrating to CA1 region.

**Temporal Pattern of CPG15 Expression and Distribution after Glutamate-Induced Injury in Primarily Cultured Hippocampal Cells**

Neuritogenesis is a critical process in re-establishment of the neural network during neurogenesis. CPG15 is known as a promoter of neuritogenesis in neuronal plasticity (Cantillops et al., 2000; Javaherian and Cline, 2005). To examine the possibility that the upregulated CPG15 may contribute to the neuritogenesis during the neurogenesis or recovery after TGI, we characterized the CPG15 expression and distribution in neuronal cells after the glutamate-induced injury of primarily cultured mouse hippocampal cells (Choi et al., 1987). The glutamate-induced injury was demonstrated using DAPI staining to show the cell nuclear fragmentation occurred after a glutamate exposure for 24 h (Fig. 3b) in comparison with the control (Fig. 3a). The analysis of CPG15 protein levels at various time points after glutamate removal showed that CPG15 expression transiently increased with a peak at 12 h (Fig. 3c,d).

In addition, we characterized the distribution of CPG15 in neuronal cells after the glutamate-induced injury using immunocytochemical staining. Figure 4-I demonstrated that CPG15 was mainly located at growth cones or in vesicles at 12 h after the injury. The location of CPG15 in the growth cones and vesicles was further confirmed using the double staining of CPG15 with neuronal markers (TUC for growth cones, VAMP for vesicles). Figure 4-II showed that CPG15 was mainly located at the ends and turn-off region of the growth cones and vesicles.

**Soluble CPG15 Is Necessary for Directing Neurite Outgrowth**

CPG15 exists as membrane anchored or soluble proteins. Recent studies have shown that soluble CPG15 is

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**FIG. 3.** Upregulation of CPG15 expression after glutamate-induced injury in primarily cultured hippocampal cells. Images (a,b) demonstrate the DAPI staining of the neurons with (b) or without (a) glutamate (Glu) stimulation in primarily cultured hippocampal cells. Scale bar = 10 μm. The arrows indicate the fragmented nuclei. Upregulation of CPG15 expression after glutamate stimulation was presented: (c) Representative Western blot images. (d) Statistical results.
an important factor in regulating embryonic brain development (Putz et al., 2005). To study the potential role of soluble CPG15 in re-establishment of the neuronal network after the glutamate-induced injury of primarily cultured mouse hippocampal cells, we detected the presence of CPG15 proteins in the media secreted from the primarily cultured hippocampal cells using ELISA. As shown in Figure 5a, the secretion of CPG15 into the media mostly occurred within the first 4 days after the injury, but not within the late 3 days, suggesting that CPG15 is secreted significantly during the early stage of the neuronal network formation.

In order to determine whether the soluble CPG15 is important in the neuronal network re-establishment, we...
added CPG15 antibodies into the culture media to deplete the soluble CPG15 and then to examine the effect of the addition of CPG15 antibodies on neurite formation of primarily cultured hippocampal cells after the glutamate-induced injury. The β-actin antibodies were added as a control. As shown in Figure 5b, the CPG15 level in the culture media was significantly depleted by adding the CPG15 antibodies, but not the β-actin antibodies.

More importantly, we found that the outgrowth of neurites reduced significantly in the media with depletion of soluble CPG15. In comparison with the control (Fig. 6A), the formation of neuronal network in the CPG15-depleted media was reduced; meanwhile, more shorter and non-targeted neurites occurred (Fig. 6B,C). Figure 6D,E statistically show the significant increases in numbers of unconnected neurites and short neurites, suggesting the requirement of soluble CPG15 in directing the neurite outgrowth during the neuronal network re-establishment after the injury.

DISCUSSION

In this report, we initiated the studies to investigate the potential role of CPG15 in re-establishment of the neuronal network after the ischemic brain injury, a process...
requiring coordinate function of multiple factors, in mouse TGI model. The results showing a transient up-regulation of CPG15 expression in the hippocampus with a peak around 14d after TGI are in consistent with the previous report that an upregulated dentate subgranular cell proliferation occurred between 1 and 2 weeks after pilocarpine-induced status epilepticus or TGI (Liu et al., 1998), indicating that CPG15 may be functionally required in recovery after the injury. This idea is further supported by analysis of the temporal profile of CPG15-positive cells and BrdU-positive cells within the same hippocampal region after the injury, showing a parallel upregulation pattern between CPG15- and BrdU-positive cell numbers in the dentate gyrus after TGI. It suggests that the upregulated CPG15 expression is likely associated with the dentate granule cell proliferation. However, the observed temporal pattern of CPG15 expression is not consistent with the function of cpg15 as an immediate-early gene (IEG) or a target of synaptic plasticity-related signal transduction pathways observed in primarily cultured cortical neurons (Fujino et al., 2003). In those cells, the CPG15 expression is induced by calcium influx through NMDA receptors and L-type voltage-sensitive calcium channels, consistent with activity-dependent regulation of CPG15 expression observed in vivo (Corriveau et al., 1999; Lee and Nedivi, 2002). Our results indicate that CPG15 may function as a promoter of neurogenesis during the recovery after an ischemic brain injury, differing from its function as an IEG in response to neural activity. Mechanisms by which CPG15 expression is up-regulated after TGI remain to be studied. It has been demonstrated that activation of the cAMP-CREB signal transport pathway increases BrdU-labeled cell numbers in the hippocampus (Nakagawa et al., 2002). CREB is known to enhance the proliferation of cells by regulating the expression of various genes in cAMP signal transport system (Duman et al., 2000). Recent studies demonstrated that CPG15 is a new member of the genes whose expression is regulated by CREB (Fujino et al., 2003). Therefore, CPG15 may act as a target gene in cAMP-CREB pathway and participate in promoting the cell regeneration in the hippocampus.

Previous studies have shown that the nascent neurons transfer from SGZ to the injury region such as CA1, and set up new neuronal network with the neurons there (Nakatomi et al., 2002). Our results demonstrate that CPG15 expression along the neurite projection in the CA1 is enhanced at 14 days after TGI. Previous studies have demonstrated that CPG15, as a factor widely expressed in neural tissues, plays critical roles in promoting neural maturation and neuroitogenesis and rescuing cortical progenitors (Cantllops et al., 2000; Javaherian and Cline, 2005). Therefore, it is likely that the potential function of CPG15 in neurogenesis during recovery after an ischemic brain injury may be related to its promoting effect on the neuronal network formation. This is supported by the results obtained in primarily cultured hippocampal cells after the glutamate-induced injury, demonstrating that during the process of neurite outgrowth or formation of neuron-neuron connections, CPG15 is mainly located at the growth cones or in the vesicles. In addition to the changes of CPG15 distribution in the cultured cells, we found that a significant amount of CPG15 is secreted into the culture media from primarily cultured hippocampal cells during the first 4 days after the glutamate injury. Moreover, by depleting the soluble CPG15 in the culture media we showed an inhibitory effect of the depletion on the neurite outgrowth of primarily cultured hippocampal cells, such as a diminishment in neuron-neuron connections and an increased formation of short and non-targeted neurites for each dendrite. The results indicate the requirement of soluble CPG15 in neuronal network formation after the injury and fit with the previous studies showing that in addition to the membrane-bound CPG15, soluble CPG15 plays a critical role in promoting neuroitogenesis (Putz et al., 2005). Together, our results suggest that both membrane-associated and soluble CPG15 proteins play important roles in neuronal network formation after the injury by promoting the outgrowth of neurites.

Up to date various factors including GPI-anchored proteins have been reported as axon guiding molecules. In Xenopus laevis, CPG15 expression is correlated with times of axon growth and synaptomature (Nedivi et al., 1998). Moreover, recent studies show that lipid rafts play very important roles in guiding axon growth cones by recruiting the axon guiding molecules (Guirland et al., 2004). These studies suggest that CPG15, as one of the membrane-associated axon guiding molecules, is most possibly involved in the process of axon guiding. Therefore, it is likely that, after TGI, in addition to the upregulation of CPG15 expression, the membrane anchored CPG15 may move towards the growth cone, and meanwhile the soluble CPG15 may be recruited to lipid rafts via unknown mechanisms. As a result, both membrane-anchored and soluble CPG15 proteins function as an axon guiding molecule to promote axonal sprouting and guide the neurite outgrowth, in coordination with other axon guiding molecules. We suspect that CPG15 depletion-caused formation of short and non-targeted neurites may be a result of inhibition of normal neurite growth due to a loss of normal axon guiding, or induction of growth of short and non-targeted neurites or both. Further studies of the related mechanisms will be important in understanding the function of CPG15 in recovery after an ischemic brain injury.
Resent studies show that CPG15 regulates the survival and apoptosis of nascent neurons in the cerebral cortex at the early developmental stage by inhibiting the apoptosis pathway promoted by caspase3 (Di Giovanni et al., 2005). The similarities in the upregulation profile of CPG15 expression at the most active stage of neural regeneration (either after TGI or at the early developmental stage of the cerebral cortex) raise a possibility that the upregulation of CPG15 expression after TGI might be an important event in relation to its function in promoting the survival of nascent cells induced by TGI via inhibiting the apoptosis-promoting effect of caspase3. This issue remains to be studied in the future.

Finding additional candidate factors enhancing the neurogenesis and studying their potential function in regulating the neuronal network re-establishment after an ischemic brain injury are important in improving the functional recovery after a stroke. In the present study, we demonstrate that CPG15 may function as a new factor required in re-establishment of neuronal network after ischemic brain injury. Further studies of molecular mechanisms by which CPG15 expression is regulated and CPG15 promotes the neuronal network re-establishment after the injury will be essential in developing a new strategy to enhance endogenous neurogenesis after the ischemic brain injury.

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