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Laboratory Investigation

Serial Expression of Hypoxia Inducible Factor-1α and Neuronal Apoptosis in Hippocampus of Rats with Chronic Ischemic Brain

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Objective : The purpose of this study is to investigate serial changes of hypoxia-inducible factor 1α (HIF-1α), as a key regulator of hypoxic ischemia, and apoptosis of hippocampus induced by bilateral carotid arteries occlusion (BCAO) in rats.

Methods : Adult male Wistar rats were subjected to the permanent BCAO. The time points studied were 1, 2, 4, 8, and 12 weeks after occlusions, with n=6 animals subjected to BCAO, and n=2 to sham operation at each time point, and brains were fixed by intracardiac perfusion fixation with 4% neutral-buffered praraformaldehyde for brain section preparation. Immunohistochemistry (IHC), western blot and terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were performed to evaluate HIF-1α expression and apoptosis.

Results : In IHC and western blot, HIF-1α levels were found to reach the peak at the 2nd week in the hippocampus, while apoptotic neurons, in TUNEL assay, were maximal at the 4th week in the hippocampus, especially in the cornu ammonis 1 (CA1) region. HIF-1α levels and apoptosis were found to fluctuate during the time course.

Conclusion : This study showed that BCAO induces acute ischemic responses for about 4 weeks then chronic ischemia in the hippocampus. These *in vivo* data are the first to show the temporal sequence of apoptosis and HIF-1α expression.

Key Words : Bilateral carotid artery occlusion · Hippocampus · Hypoxic ischemia · Hypoxia-inducible factor 1α · Apoptosis.

INTRODUCTION

Various kinds of animal model such as transient and chronic ischemia model have been developed to study the pathophysiology and the efficacy of various treatment modalities[6,11,26]. These types of ischemia model are showed the temporal changes of cerebral blood flow (CBF) and various degrees of infarctions that produce brain damage[12]. Brain damage progresses and ultimately becomes irreversible unless the oxygen supply is restored. The most vulnerable areas seem to be the hippocampus and cerebral cortex[18].

Bilateral carotid artery occlusion (BCAO) of rat is chronic ischemia model, which induces consistent alteration of cerebral metabolism and ischemic brain damage. This method has been used to lead moderate CBF reduction, falling into the chronic ischemia range in adult rat brains[4,9,15,20,23]. BCAO is tolerated by adult rats due to effective collateral flow through the posterior communicating arteries and the arterial circle of Willis. However, BCAO induces morphological abnormalities and quantifiable cell loss in hippocampus[1,8,24]. In rat brain with BCAO, neuronal cell loss occurs mainly through necrosis, and apoptosis as well[18,19]. However, the temporal change of apoptosis has not been clarified in this model.

Hypoxic ischemia induces the expression of hypoxia inducible factor 1 (HIF-1)[2,28] and its level can remain elevated for a few weeks. HIF-1 is a heterodimer composed of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit[32,33] HIF-1α is continuously produced and degraded under normoxic condition but under hypoxic condition, HIF-1α degradation is inhibited, resulting in its accumulation[29]. HIF-1α plays a pivotal role in cellular oxygen homeostasis by regulating the expression of genes including glycolysis, erythropoiesis, and angiogenesis[28]. Recently there has been increasing interest in an ischemic condition resulting in neuronal loss that may be mediated via apoptosis[14]. As a key regulator of hypoxia, HIF-1α expression may regulate apoptosis induction[13]. It is currently unknown whether HIF-1α levels change during chronic ischemia after BCAO.

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In this study, immunohistochemistry (IHC), western blot and terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to examine the temporal changes of apoptosis and HIF-1α expression in the hippocampus following BCAO in the rat.

MATERIALS AND METHODS

Chronic hypoxic ischemia

Adult male Wistar rats (body weight 280-350 g, about 10 weeks of age) were anesthetized with 3% isoflurane in 70% N_2O and balance of O_2. BCAO was performed as described in the literature[14,16,27]. Briefly, following a midline incision, both common carotid arteries were exposed. The common carotid arteries were separated from the vagal nerves and BCAO was performed with 3-0 silk. After occlusion, all animals appeared overtly normal and did not display grossly abnormal behavior or seizures. Sham operation (surgery without BCAO) was used as a control. The time points studied were 1, 2, 4, 8 and 12 weeks after occlusion, with n=6 animals subjected to BCAO, and n=2 to sham operation at each time point. After different survival periods, brains were fixed by intracardiac perfusion fixation with 4% neutral-buffered praraformaldehyde for brain section preparation[7]. Serial coronal sections were cut at 4 μm. Sections located -3.3 to -4.3 from the bregma were analyzed. The protocols were approved by the Animal Care and Use Committee. The study was designed to minimize the number of animals required, and all efforts were made to minimize suffering.

In situ TUNEL staining for quantification of apoptotic neurons

Paraffin sections (4 μm thick) were placed on Superfrost/plus slides, deparaffinized, and rehydrated. Briefly, sections were first treated with 20 μg/mL of proteinase K in 0.1 M PBS (pH 7.4) for 20 minutes at 37°C. After washing in phosphate-buffered saline (PBS), sections were covered with 50 μL of the TUNEL reaction mixture (Roche, Basel, Switzerland) which contained TdT and fluorescein-dUTP, and incubated under a coverslip in a humidified chamber for 1 hour at 37°C. The sections were then incubated with an anti-fluorescein-alkaline phosphatase conjugate diluted 1/3 in 100 mM Tris-HCL, 150 mM NaCl (pH 7.5), and 1% blocking reagent for 1 hour. After three 15 minute washes in PBS, sections were stained for 5-15 minutes and counterstained with 0.5% methyl green.

Immunohistochemistry

Immunohistochemical analysis of the brain sections was performed using the two step Envision system (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The primary antibody was HIF-1α (monoclonal, dilution 1 : 200, abcam, Cambridge, MA, USA). In brief, tissue sections were first treated with 5% H_2O_2 in PBS for 20 minutes at room temperature to quench endogenous peroxidase, followed by three PBS washes. HIF-1α antigen retrieval was performed by heating slides in citric acid buffer (pH 6.0) in a microwave oven (high power) for 10 minutes. After three 3-minute washes with PBS, sections treated with antibodies of a mitochondrial marker were incubated in a blocking solution of 5% normal goat serum for 30 minutes. Primary antibody was incubated overnight at 4°C. Slides were visualized by means of the two step Envision system- alkaline phosphatase. The slides were counterstained with Harrison's hematoxylin and coverslipped.

Western blotting

Animals were sacrificed 1, 2, 4, 8 and 12 weeks after BCAO and sham operation. Pooled (n=3 per group) hippocampus samples were homogenized and lysed in buffer containing 1% Nonidet P-40 and the protease inhibitors. Lysastes were cleared by centrifugation, and protein concentration was determined spectrophotometrically at A^{595} nm using the Bradford reagent. Samples (50 μg) were boiled in gel loading buffer and then separated on 12% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes (Pall life science, Hampshire, UK) and incubated with HIF-1α (dilution 1 : 300, abcam, Cambridge, MA, USA). The membrane was then incubated with the appropriate secondary antibody (1 : 15000 dilution, cell signaling, Danvers, MA, USA), followed by chemiluminescence detection (Gene depot, Seoul, Korea), and exposed to Agfa Film.

Image analysis

HIF-1α and TUNEL stained cells of Cornu Ammonis fields 1 (CA1), Cornu Ammonis fields 3 (CA3), and dentate gyrus (DG) were examined and counted under 100× magnification. To quantify the positive cells, staining images were collected using an Olympus BX41 microscope fitted with a Leica DFC 290 digital camera and analyzed using Image Pro Plus software (Media cybernetics Inc., Bethesda, MD, USA).

Statistical analysis

The data were expressed as means±SEM using SPSS software. Significance was determined by a one-way ANOVA, followed by Fisher's LSD post-hoc test for multiple comparisons. p-value of less than 0.05 was regarded as statistically significant.

RESULTS

TUNEL staining

One week after BCAO, TUNEL-positive cells were observed in the CA1 region. These positive neurons were greater than those observed in the corresponding regions of sham-operated rats. There were more TUNEL-positive cells in the CA1, CA3 and DG at the 2nd weeks compared to the 1st week after BCAO. Apoptotic neurons were increased at the 4th week compared to the 2nd week after BCAO. TUNEL-positive cells were decreased in the CA1 and DG region at the 8th week compared to the 4th

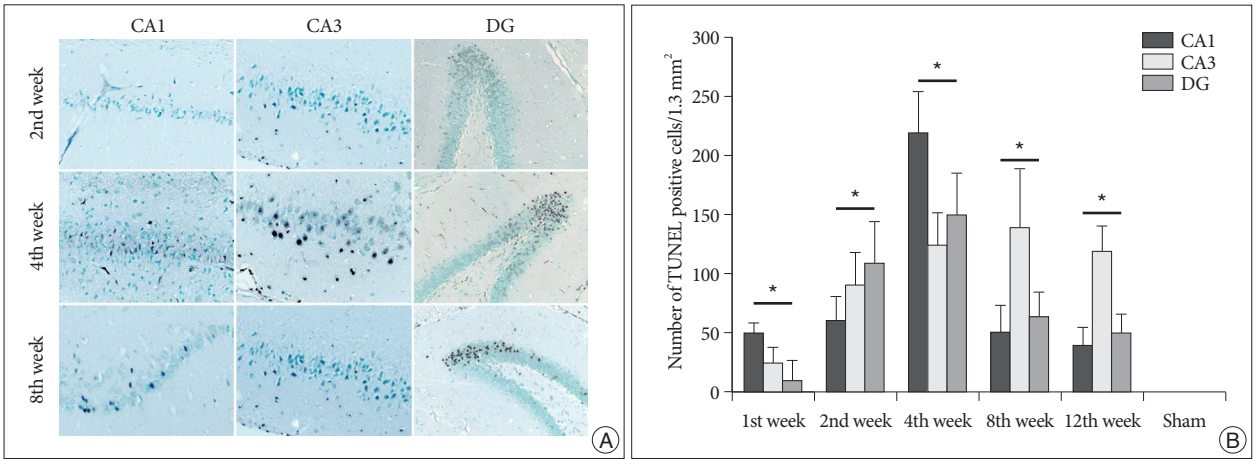

Fig. 1. A : TUNEL staining of the hippocampus at the 2nd week, 4th week and 8th week. B : The bar graph showing the number of TUNEL positive cells in various regions of the hippocampus. Total number of TUNEL positive cells are maximal at the 4th week. Changes in number of TUNEL positive cells are statistically significant (*) all the time. TUNEL : terminal uridine deoxynucleotidyl transferase dUTP nick end labeling, CA : cornu ammonis, DG : dentate gyrus.

week, although in the CA3 region, it had increased a little bit more (Fig. 1A). At the 12th week after BCAO, TUNEL-positive cells also were observed. No TUNEL-positive cells were detected in any brain region of sham-operated rats (data not shown). Quantification showed that total number of apoptotic neurons was increased until the 4th week after BCAO in the hippocampus (Fig. 1B).

Serial expression of HIF-1α in the hippocampus

HIF-1α expression was examined using Western blot analysis. HIF-1α expression was observed from the 1st week after BCAO, and increased time-dependently until reaching the peak at the 2nd week after BCAO, the time after which the HIF-1α levels decreased. At the 12th week, HIF-1α expression showed normal levels as shown in sham-operated rats (Fig. 2). Quantification showed there was a statistical difference between HIF-1α levels at the 1st, 2nd and 4th week after BCAO and in sham-operated rats (Fig. 2B).

HIF-1α expression was also examined to analyze region of positive cells using IHC assays. At the 1st week after BCAO, HIF-1α expression appeared in the CA1, CA3, DG region. At the 2nd week after BCAO, many neurons were densely stained, with some neurons showing high expression compared with those at the 1st week (Fig. 3A). After 2 weeks, HIF-1α expression decreased markedly in the hippocampus. At the 12th week, HIF-1α levels were similar to those observed in the sham-operated group. Quantitative data of the IHC (Fig. 3B) were consistent with those of the western blot studies in the hippocampus, with both assays showing peak HIF-1α expression at the 2nd week after BCAO.

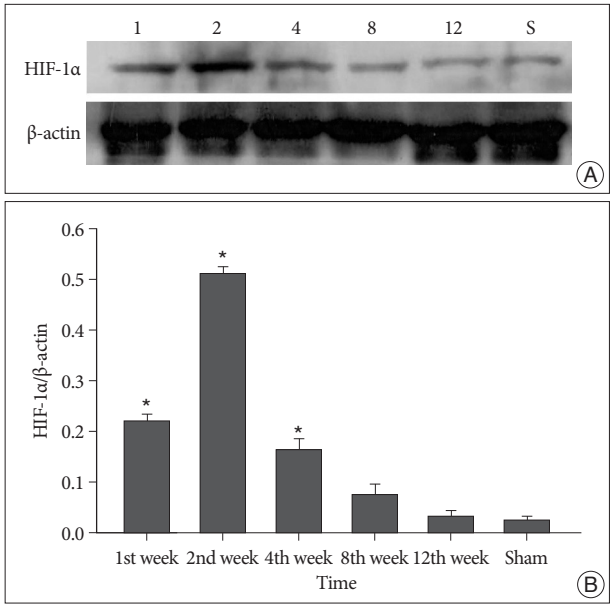

Fig. 2. HIF-1α western blot (A) and densitometric analysis (B) in the hippocampus. Western blot analysis of the hippocampus shows that expression of HIF-1α was maximal at the 2nd week. HIF-1α expression is statistically significant (*) in the 1st, 2nd, and 4th week. 1, 2, 4, 8, 12 means corresponding week after BCAO respectively. HIF-1α : hypoxia-inducible factor 1α, BCAO : Bilateral carotid artery occlusion.

DISCUSSION

In human cerebrovascular insufficiency, impaired vasoreactivity has been used as an indicator of increased stroke risk. Ulrich et al.[31] demonstrated decreased vasoreactivity in this rat model of BCAO. Therefore, this model allows to investigate the hemodynamic insufficiency in a similar setting in human conditions such as carotid occlusion or Moya Moya disease.

This study aimed to clarify the temporal sequence of apoptosis and HIF-1α expression in the hippocampus in a rat model of chronic ischemia. This model is distinct from experimental paradigms of transient global ischemia. In transient models, where ischemia is induced by cardiac arrest and resuscitation, HIF-1α starts to accumulate in as early as 1 hour of recovery, peaks on the first day, and persists for 7 days. HIF-1α is no longer present

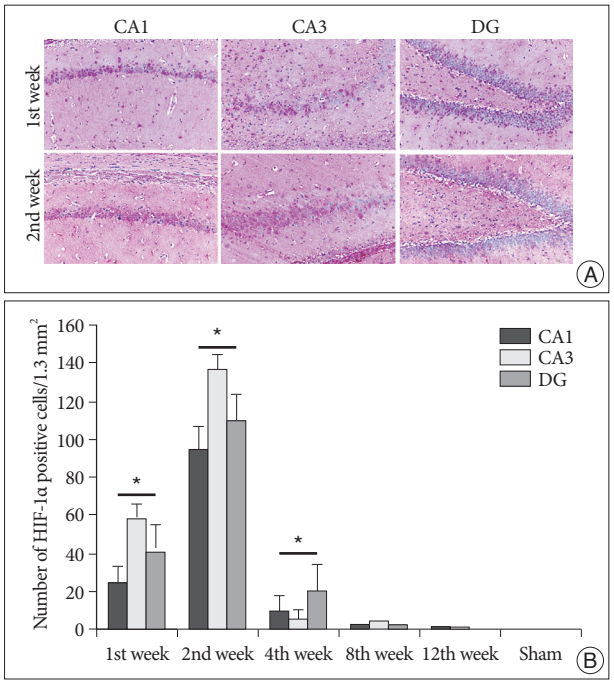

Fig. 3. HIF-1α immunohistochemisty (A) and quantitative analysis (B) shows that the number of HIF-1α positive cells decreased rapidly after the 2nd week. Changes in numbers of HIF-1α positive cells are statistically significant (*) in the 1st, 2nd, and 4th week. HIF-1α : hypoxia-inducible factor 1α, CA : cornu ammonis, DG : dentate gyrus.

after 2 weeks[3]. In contrast, our study showed that HIF-1α protein levels in the hippocampus reached its peak on the second week after BCAO. These findings suggest that HIF-1α degradation was inhibited, leading to protein accumulation up to 2 weeks. Under conditions of reduced O_2 availability, HIF-1α degradation is inhibited, which ultimately leads to increased transcription[17,25]. It can be assumed that sudden reduction of CBF induced by BCAO leads to increased expression of HIF-1α. Recovery of CBF via compensatory and adaptive mechanisms might normalize HIF-1α expression over the weeks or months.

IHC in this study showed that HIF-1α was expressed in neurons at 1 week after BCAO in the CA1, CA3 and DG and that expression further increased up to 2 weeks. There were similar numbers of immunoreactive neurons in the CA1, CA3 and DG regions at the first week, indicating that these regions were simultaneously affected by ischemia. After 4 weeks, HIF-1α expression in the hippocampus was similar to that in sham-operated rats. We believe that acute ischemic responses were subsided from the 4th week due to cellular adaptation and CBF restoration. Previous study reported that diameter of capillaries and expression of vascular endothelial growth factor increased progressively after BCAO[21]. Farkas et al.[10] reported that compensatory blood flow might be provided through artery dilation, the recruitment of non-perfused capillaries and angiogenesis in BCAO rat.

It has been known that two types of brain damages are observed in the rat with BCAO[19]. One type is the infarction, observed in the cerebral cortex and striatum. Another type is progressive neuronal damage including apoptosis in the hippocampus and white matter. BCAO induced neuronal damage can be visualized with conventional methods such as hematoxylin & eosin, toluidine blue, cresyl violet or with more sophisticated technique such as the TUNEL assay.

Tomimoto et al.[30] reported that the number of apoptotic neuron was peak in the white matter and gray matter at 2 weeks after BCAO. After those time points, apoptosis decreased markedly, indicating that compensatory or adaptive mechanisms such as restoring of CBF are worked in the brain. It is reported that CBF normalizes over a several months after BCAO induction[5,22]. CBF restoration may be provided through vessel dilation, the recruitment of nonperfused capillaries and angiogenesis. Oldendorf et al.[22] showed that diameter of the posterior vessels supplying to the circle of Willis were increased significantly at 12 weeks to 6 months after BCAO induction. Bennett et al.[1] showed that BCAO induced apoptotic neuronal cell deaths which are apoptotic in morphology and DNA strand breaks in hippocampal neuron, by using TUNEL assay. The study showed that apoptosis of neuron was detected specifically in the hippocampus including CA1, CA2, CA3 and other regions at the second week and it persisted until the 27th week[1]. The amount of apoptosis had further increased in the 27th week than in the second week in CA1 and especially in CA3 region, whereas in other regions it had decreased. In our study, the number of total apoptotic neuron in the hippocampus was at its peak at the 4th weeks after BCAO. However, in CA3 region, apoptotic neurons had increased until the 8th week then somewhat decreased. It was not statistically significant though. The progression after the 12th week was not covered in our study.

CONCLUSION

According to the changes of HIF-1α and apoptosis, it is presumed that the process of acute ischemia in the hippocampus induced by BCAO sustains for about 4 weeks which then turns to the process of chronic ischemia afterwards. Their changes were found to fluctuate during the time course. These *in vivo* data are the first to show the temporal sequence of HIF-1α expression and apoptosis.

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