Does Hypoxic Preconditioning Induce Angiogenesis and Protect against Focal Cerebral Ischemic Damage in Rats?

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Background and Aims : This study investigated whether hypoxic preconditioning (HP) induces ischemic tolerance in an established experimental model of permanent middle cerebral artery occlusion using rats. **Methods :** Animals were divided into the normoxia (control) and HP groups.   HP was performed under normobaric conditions (7% O_2 for 2 hours), 14 days before focal cerebral ischemia induction.   The infarct volume was evaluated by quantitative histopathology.   Immunofluorescence analysis was assessed at 24 hours after HP to examine the effect on proliferating cell and microglia/macrophage activation. Brain microvessel density was assessed 14 days after HP.   Furthermore, the expression was evaluated of angiogenesis-related proteins such as hypoxia-inducible factor-1α and vascular endothelial growth factor proteins.   **Results :** Hemisphere infarct volume in the HP group was significantly smaller than in the control group.   Vascular endothelial growth factor and proliferating cell nuclear antigen expression was not increased after HP.   HP tended to increase vessel density relative to the control group, but this did not achieve statistical significance.   **Conclusion :** HP attenuated the infarct volume in rats.   The phenomenon of ischemic tolerance may link HP with vascular remodeling. (Kitakanto Med J 2011 ; 61 : 1 ～ 8)

Key words : hypoxia, preconditioning, tolerance, endothelial cell, angiogenesis

Introduction

The brain is the most oxygen-dependent organ of the body, so is intrinsically more vulnerable to ischemia than any other organ.[1-3]   A few minutes of cerebral ischemia is sufficient to generate significant damage to brain, whereas 20–40 minutes of ischemia is needed to cause ischemic injury to heart and kidney.[4] Every organ of the body has an intrinsic defense system against ischemic stress.   Ischemic tolerance is one of the adaptive processes inducing resistance to subsequent ischemic events.[1]   Ischemic tolerance was first described in the heart,[5] and was first demonstrated in the brain using the gerbil model of global ischemia.[6,7] This phenomenon has since been widely identified in many other animals, models of global and focal is-chemia, and using other preconditioning challenges including sublethal hypothermia, hyperbaric oxygen, metabolic inhibition, and cytokines.[4]   Experiments have established that ischemic preconditioning is a transient phenomenon that takes at least 24 hours to develop after the first brief episode of transient ischemia, lasts approximately 1 week, and disappears by 2 weeks.[8]

Similar phenomena have been identified in stroke patients who showed acquired ischemic tolerance after transient ischemic attack (TIA) occurring prior to the symptomatic ischemia.[9,10]   However, the clinical interval between the TIA and subsequent stroke ranged from a few hours to years.[11]   The timing of the TIA seemed to have no impact on the severity of the subsequent stroke.   Therefore, several different mechanisms

may be involved in the induction of ischemic tolerance in humans, in addition to the mechanism identified by experimental studies.   In particular, the possibility of upregulation in the collateral circulation must be considered.[12]

The present study investigated whether hypoxic preconditioning (HP) induced 14 days prior to the ischemic insult affected ischemic tolerance in an established experimental model using permanent middle cerebral artery (MCA) occlusion.   The mechanism of action of ischemic tolerance was investigated by assessing vascular remodeling after HP to identify any endogenous response of the vascular system possibly involved in the development of ischemic tolerance.

Materials and Methods

Male Wistar rats (300 to 330 g) were obtained from Charles River Japan (Tsukuba, Ibaraki, Japan). Animals were housed in individual cages in a temperature and light controlled environment and allowed free access to chow and water.   All procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Gunma University, Faculty of Medicine.   All treatments and analysis were performed by surgeons and investigators unaware of the group identity of any animals.   Fig. 1 summarizes the experimental protocols.

HP treatment

Normobaric hypoxia (7% O_2/93% N_2 for 2 h) was achieved by replacement of oxygen by nitrogen in a sealed chamber (1.5m,^{3} P-5100S type ; Barotec Hanyuda Co., Tokyo, Japan) in which oxygen fraction, temperature, and hygrometry were continuously controlled.   Control animals were maintained at 37°C for 2 hours under normoxic conditions in the chamber. Exposure of the rats to hypoxia caused increased respiratory rate and decreased locomotor activities, but all animals immediately recovered normal respiration rate and behavior after release from the chamber.   No weight loss was observed at any time after hypoxia and no animals showed visible neurologic deficits such as hemiparesis or epilepsy.   No mortality occurred during the HP.
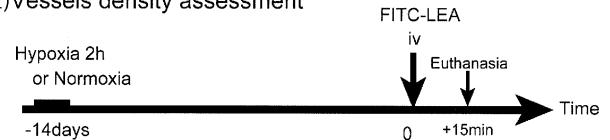
Induction of focal cerebral ischemia

All animals were anesthetized with isoflurane 1.5% in nitrous oxide-oxygen (70 : 30), then intubated and artificially ventilated.   The femoral artery was cannulated for recording arterial pressure and blood gases, and temperature was controlled with a rectal thermometer and a heating pad.   Rats were maintained normotensive, normocapnic, and normothermic during anesthesia.   Focal cerebral ischemia was in-
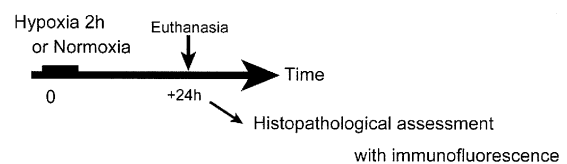

Fig. 1 Schematic representations of the ischemia studies related to hypoxic preconditioning (HP).   HP was expected to induce ischemic tolerance stepwise, so experiments were performed over various time courses.   1) Infarction volume assessment ; 2) Cerebral microvessel density assessment by FITC-LEA administration ; 3) Histopathological assessment of the brain tissue exposed to hypoxia or normoxia.

duced using the permanent MCA occlusion method[13] with some modifications to ensure reproducible infarction.[14]   Briefly, a 2-cm skin incision was made on the left temporal region, followed by a small subtemporal craniectomy to expose the left MCA from the proximal segment passing over the olfactory nerve as far as the distal part intersecting the inferior cerebral vein. Cerebral ischemia was then induced by electrocoagulation of the MCA from a point proximal to the origin of the lenticulostriate artery to the point where the artery crosses the inferior cerebral vein.   The MCA was then transected at the level of the olfactory tract to confirm complete occlusion of the artery.   The time of transection was taken as the exact time of MCA occlusion.   Then, the skull window was left open and the muscle and skin were sutured.   Body temperature was monitored and maintained until full recovery of all animals.

Infarct volume assessment

Rats were euthanized 24 hours after MCA occlusion and the brains were rapidly removed and sliced coronally at 1-mm intervals.   These brain sections were stained with 2, 3, 5-triphenyltetrazolium chloride

(Sigma-Aldrich, St Louis, MO, USA), as described previously.[15] The areas of ischemic infarction were transcribed onto scale diagrams of 8 predetermined coronal planes of the forebrain.[16] Infarct areas on the diagrams were then measured with an image analyzer (Adobe Photoshop software; Adobe Systems Inc., San Jose, CA, USA) and integrated using the known distance between each coronal level to determine the total volume of ischemic damage in each brain. Immunofluorescence analysis of proliferative cells and microglia

Brain sections were prepared at 24 hours after HP. Briefly, the rats were deeply anesthetized with 5 % isoflurane, placed in the supine position, and the thorax opened through bilateral incisions. A catheter was inserted into the left ventricle of the heart and the right atrium was incised, then heparinized saline was infused at a pressure equal to the arterial blood pressure (90 to 110 mmHg) until the perfusate from the right atrium was bloodless. The heparinized solution was followed by 300 ml of 4 % paraformaldehyde in phosphate buffered saline (PBS). The brains were removed and postfixed in the same solution for 24 hours, and then immersed in 30% sucrose solution until no longer buoyant. Samples were embedded in Tissue-Tek O.C.T. (Sakura Finetek Inc., Torrance, CA, USA) and frozen at −80°C. Rostro-caudal coronal sections (20 μm) at multiple levels were cut on a cryostat. Sections were mounted on amino-silane-coated slides and dried at room temperature. Sections were microwaved for 10 minutes in 10 mmol/l citrate acid, pH 6.0, and allowed to cool to room temperature for 60 minutes. Sections were incubated in blocking buffer (0.2% Triton X-100/0.1% bovine serum albumin in PBS) for 1 hour at room temperature, then incubated in a humidified chamber with a mixture of two primary antibodies (polyclonal anti-glial fibrillary acidic protein [GFAP] [Z0334; Dako Denmark A/S, Glostrup, Denmark] and monoclonal anti-proliferating cell nuclear antigen [PCNA] [clone PC10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] or polyclonal anti-GFAP [Z0334; Dako Denmark A/S] and monoclonal anti-ED1 [clone JC/70A; Dako Denmark A/S]) diluted with PBS (GFAP, 1:1000; PCNA, 1:100; ED1, 1:500). Sections were analyzed with a fluorescent microscope and images captured through a 20X objective and 10X eyepiece lens. Images were saved for subsequent analysis and quantification using Adobe Photoshop software (Adobe Systems Inc.) Immunostained samples for ED1 and PCNA were analyzed by positive cell counting in 1-mm² microscope fields (at 200X magnification) in the cortex and caudate nucleus. The counting threshold was set at the signal intensity of the most intensely

stained cells in the contralateral internal capsule. The immunopositive cells were quantified by image software (Image J 1.36b; NIH, Bethesda, MD, USA). The number of ED1-and PCNA-immunopositive cells was quantified as the mean cell counts in nine randomly selected regions of interest in each cortex or caudate nucleus.

Intravascular lectin administration

To assess the effect of HP on vascular structures, animals were intravascularly labeled using 100 μg of fluorescein isothiocyanate (FITC)-conjugated Lycopersicon esculentum agglutinin (LEA) lectin (FL-1171, 2 mg/ml; Vector Laboratories Inc., Burlingame, CA, USA). Microvessel staining and counting were performed as previously described.[17] Briefly, rats were deeply anesthetized as described above, and FITC-LEA was delivered systemically by an intravenous injection into the surgically exposed right femoral vein. A volume of 250 μl of FITC-LEA was administered to each rat at 1.2 ml/h using a syringe pump (model no. 780100; KD Scientific, Holliston, MA, USA). FITC-LEA lectin was allowed to circulate for 15 minutes before perfusion with saline. The brains were rapidly removed, postfixed in the same solution for 4 hours, and then immersed in 30% sucrose solution until no longer buoyant. The brains were then embedded in Tissue-Tek O.C.T. compound. Rostro-caudal coronal sections (10 μm) were cut on a cryostat. Blood vessels were defined morphologically, and counted in 1-mm² microscope fields (at 200X magnification). Vessels with a diameter between 3 and 8 μm were counted. The numbers of microvessels were calculated as the mean of the blood vessel counts obtained from three microscopic fields in randomly selected regions of interest in each cortex or caudate nucleus.

In addition, mouse monoclonal antibody against hypoxia inducible factors HIF-1α (H1alpha 67; Novus Biological, Littleton, CO, USA) (1:500 dilution) and rabbit polyclonal antibody against vascular endothelial growth factor (VEGF) (sc-507; Santa Cruz Biotechnology, Inc.) (1:100 dilution) were used to detect HIF1-α and VEGF. Sections were washed in PBS and then incubated with a mixture of two fluorescent secondary antibodies (Alexa Fluor 488 [A11070; Molecular Probes Europe BV, Leiden, the Netherlands] goat anti-rabbit IgG and Alexa Fluor 594 [A11020; Molecular Probes Europe BV] goat anti-mouse IgG) diluted with PBS (Alexa Fluor 488, 1:2500; Alexa Fluor 594, 1:2500). Sections were analyzed with a fluorescence microscope and images captured through a 20X objective and 10X eyepiece lens.

Statistical analysis

All data are presented as mean ± standard deviation. Comparison between groups was carried out using the χ^2 test, Bartlett test, or analysis of variance followed by Tukey-Kramer tests as indicated in the text. All values were considered to be significant at $P < 0.01$.

Results

Effect of HP on infarct volume

Mean volume of infarction in the cerebral hemisphere was 87.6 ± 14.8 mm³ in the control group, and 66.0 ± 7.3 mm³ in the HP group. Mean volume of infarction at cerebral cortex was 61.0 ± 13.6 mm³ in the control group, and 54.6 ± 11.3 mm³ in the HP group. Mean volume of infarction at caudate nucleus was 24.2 ± 3.5 mm³ in the control group, and 11.9 ± 4.2 mm³ in the HP group (Fig. 2A, B). Therefore, HP significantly reduced infarction volume in the cerebral hemisphere and the caudate nucleus (control group, n=9 ; HP group, n=8 : both $P < 0.01$).

Monitoring of rectal temperature, blood gases, pH, and mean arterial pressure during MCA occlusions showed no differences between the groups (data not shown), so the neuroprotective effect cannot be explained by changes in the systemic cardiovascular and biochemical parameters at the moment of focal ischemia.

Effect of HP on proliferating cell and microglia/macrophage activation

To verify the responsiveness of microglia to brain tissue hypoxia, ED1 immunofluorescence was compared in control rats at 24 hours after exposure to either normoxia or hypoxia. Immunohistochemistry showed ED1-immunopositive cells were barely detectable in both groups in the cortex and caudate nucleus (control group, n=6 ; HP group, n=6) (Fig. 3A, B). Examination of cell proliferation by immunostaining with PCNA, an intranuclear acidic protein highly expressed in proliferating cells, showed PCNA-immunopositive cells were rarely observed in the small vessels of both groups (control group, n=6 ; HP group, n=6) (Fig. 4A, B).

Effect of HP on angiogenesis

To determine whether HP induced brain angiogenesis, microvessel density was measured after
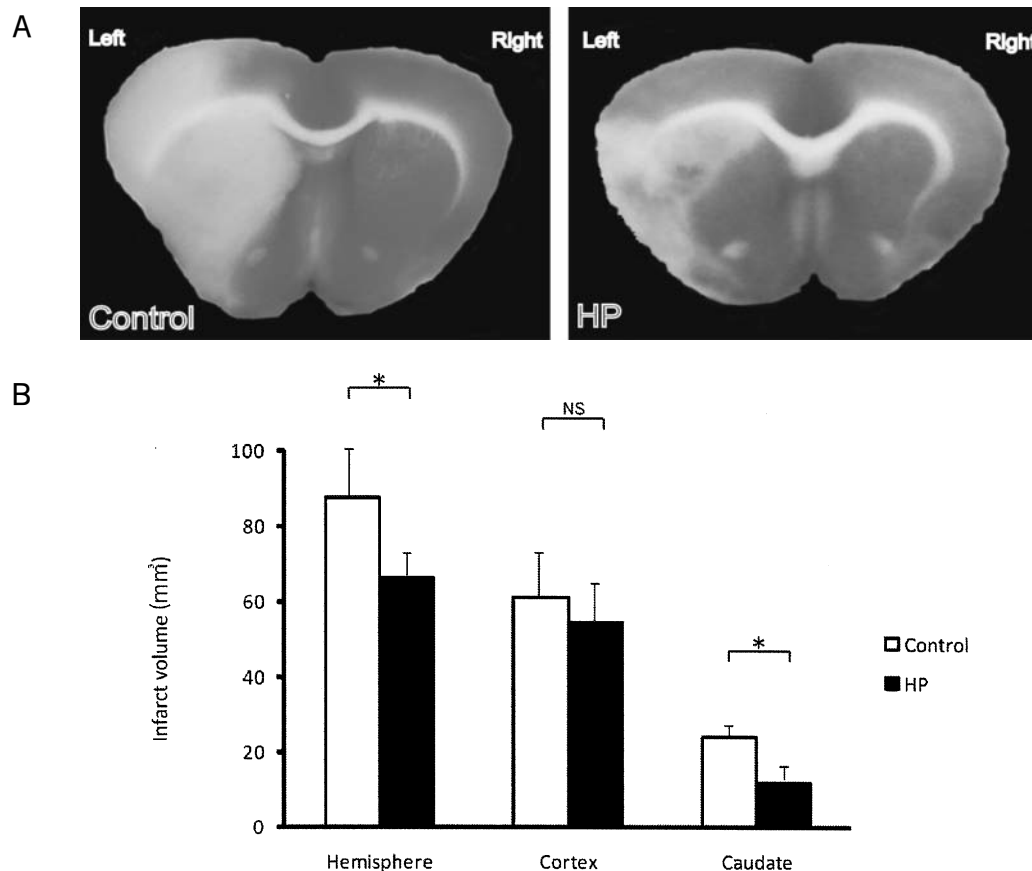

Fig. 2 A : Infarct volume was determined with 2, 3, 5-triphenyltetrazolium chloride staining at 24 hours after MCA occlusion. Infarction volume was significantly reduced in animals that underwent HP compared to control animals.

B : Statistical analysis of infarction volume : Infarct volume was reduced in animals exposed to HP ($*P < 0.01$).
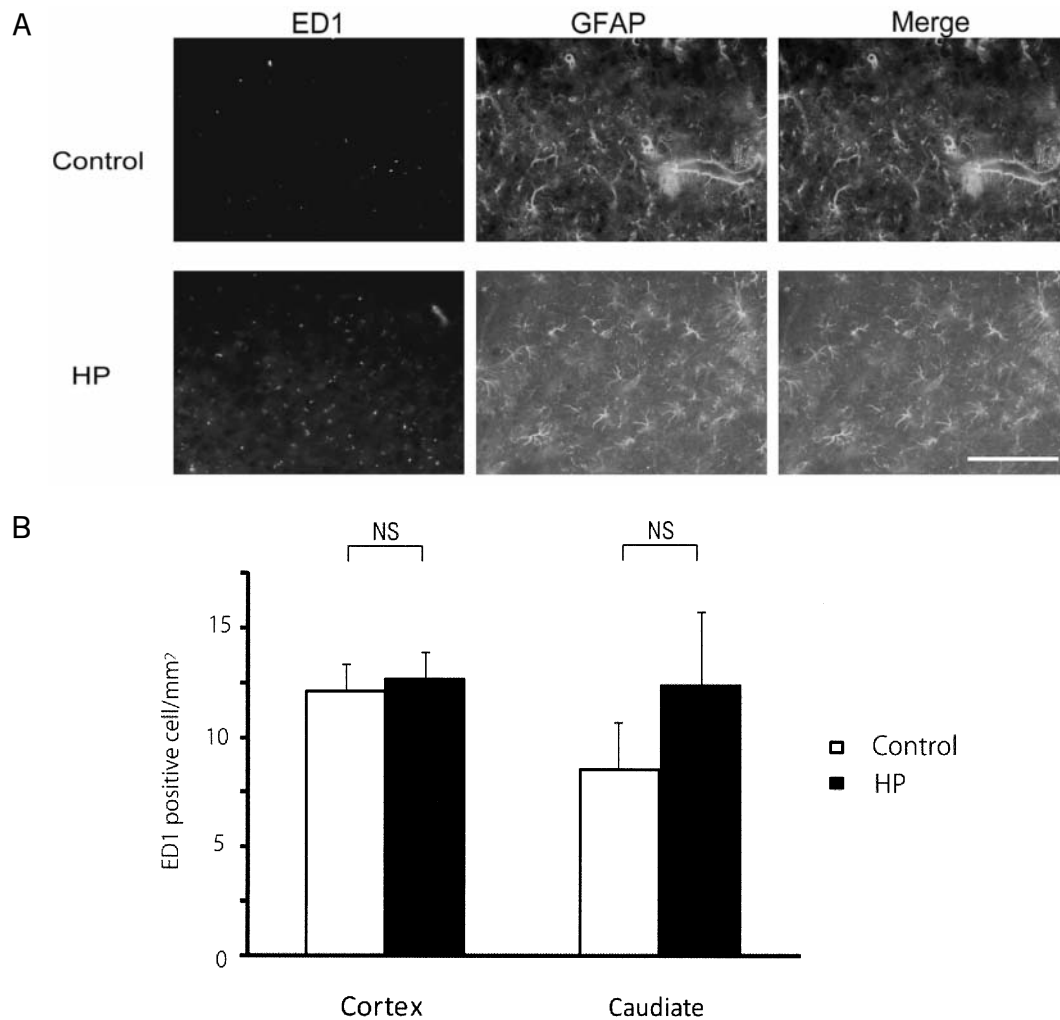
A

| | ED1 | GFAP | Merge |

Control

HP

B

Fig. 3 A : Double-labeled immunofluorescence staining for ED1 (red) and GFAP (green) was performed to evaluate changes at cortex and caudate nucleus. ED1-positive cells were barely detected in the control and HP groups. GFAP staining of astrocytes did not show remarkable changes in any group. Merged images show morphological changes at the perivascular sites in microglia/macrophages. Scale bar＝50 μm.

B : Quantification of microglia counted all ED1-positive cells in 1 mm² of cortex and caudate tissue.

perfusion with a FITC-labeled vascular lectin. The number of microvessels contained in 1 mm² of cortex for the HP group (n＝4, 9.5 ± 1.6/mm²) was not significantly increased compared with the control group (n＝5, 8.1 ± 0.8/mm²) (Fig. 5A, B). In the study, HP tended to increase vessel density relative to control, but this did not achieve statistical significance.

Furthermore, HIF-1α and VEGF, which are associated with angiogenesis, were expressed in all rats (control group, n＝4 ; HP group, n＝5) (Fig. 6). The immunofluorescence signals of these proteins were observed predominantly in the cytoplasm of cells presumed to be neurons, with fewer signals in the microvessels and astrocytes. No quantification of positive cells was undertaken due to the difficulty in defining a threshold value. However, the fluorescence intensity of cells in rats after HP appeared to be stronger than in control rats.

Discussion

The principal finding of our study is that infarct volume at 24 hours after permanent MCA occlusion in rats was significantly reduced by HP performed 14 days previously compared to the control group. Collateral supply through small blood vessels may determine the severity of ischemic injury after occlusion of MCA in stroke patients.[18] Therefore, we hypothesized that the higher density of blood vessels constructed by vascular remodeling after hypoxic stress has a neuroprotective effect in focal cerebral ischemia. To explore the mechanism of neuroprotection, histological examination at 14 days after HP revealed a relative increase in the number of small blood vessels in both the caudate nucleus and cortex of HP rats compared with the control group. This finding might indicate part of the cause of the neuroprotection, but the experiment has
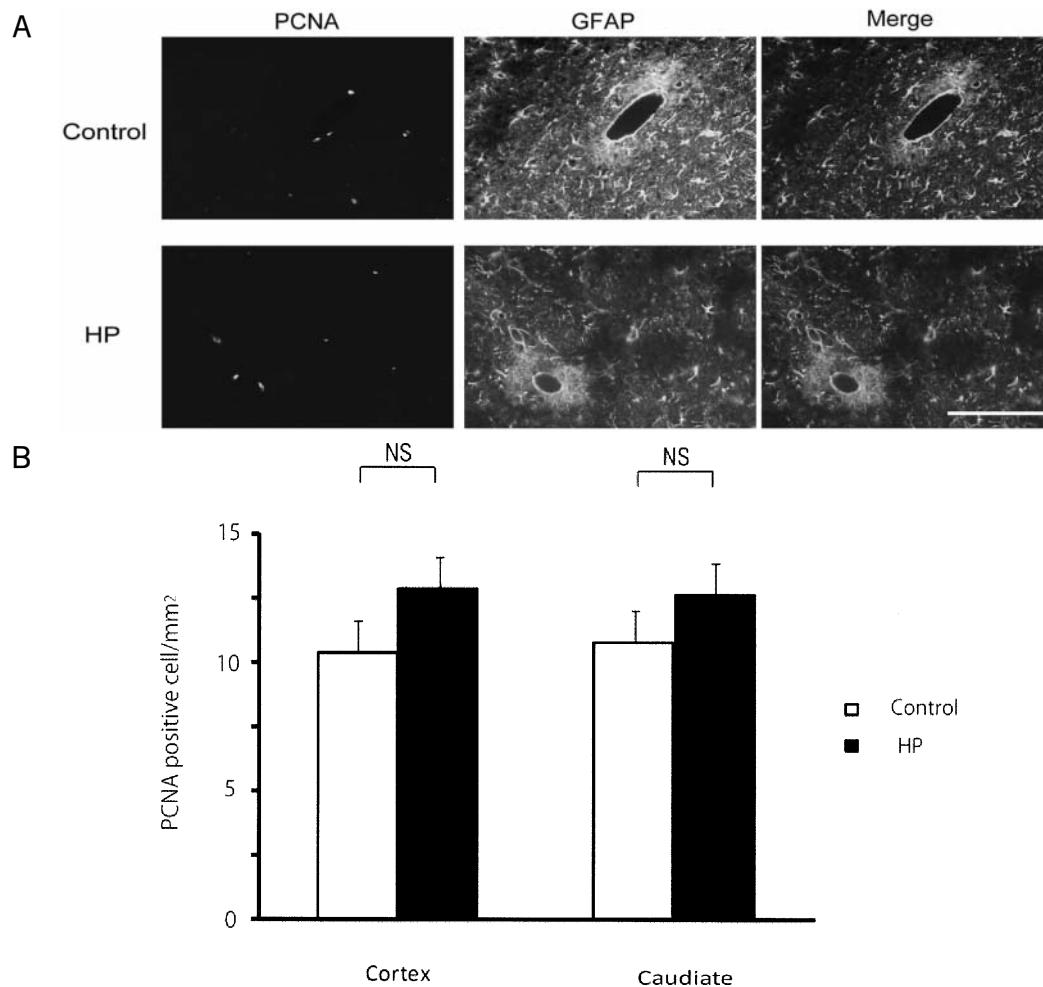
Fig. 4 Proliferative cells and astrocytes.

　　　A : Double-labeled immunofluorescence staining for PCNA (red) of proliferative cells and GFAP (green)
　　　　 of astrocytes.　Merged images show morphological changes at the perivascular sites in proliferating
　　　　 cells.　Scale bar＝50 μm.

　　　B : PCNA-positive cell count in 1 mm² was not significantly increased in the HP group compared with the
　　　　 control group.

failed to show robust proof that HP induces the vascular remodeling.

　　We explored what pathological changes were evident immediately after HP in vivo, to try to identify the stimulation mechanism for the formation of new blood vessels.　Firstly, we focused on the histopathology, since HP is important in promoting morphological changes that will confer more resistance in the brain tissues to subsequent ischemic events.　We evaluated such changes in proliferating cells and microglia/macrophages as well as expression of hypoxia-related proteins such as HIF-1α and VEGF proteins in the in vivo study.　Under hypoxic conditions, the master transcriptional regulators of both cellular and systemic adaptation are the HIF-1α family.[19]　We confirmed HIF-1α expression after HP with histopathology, but did not quantify HIF-1α expression due to difficulties in setting a threshold value.　However, increased HIF-1α expression seems to be relatively clear in rats with HP compared to the control

group.　HIF-1α subunits are degraded rapidly by 26s proteasomes under normoxia, whereas HIF-1α is stabilized and transactivated to the nucleus during hypoxia.[20]　HIF-1α which escapes degradation binds to the constitutively expressed HIF-1β, and exerts its hypoxic response through binding to the cis-consensus HIF-1-binding site, the hypoxic-responsive element.[1,4] The role of oxygen tension in regulating VEGF levels is critical to its function as an angiogenic factor. Accumulation of VEGF messenger ribonucleic acid is induced by exposure to low oxygen levels both in vitro and in vivo.[21]

　　Several limitations of this study must be addressed.　We did not investigate other HP hypotheses, such as inhibition of excitotoxicity,[22] so we cannot exclude the potential involvement of other signaling pathways in the development of ischemic tolerance. We have still to identify the optimum HP parameters : degree, duration and timing of HP.　Indeed, we cannot fully explain the mechanism of ischemic tolerance
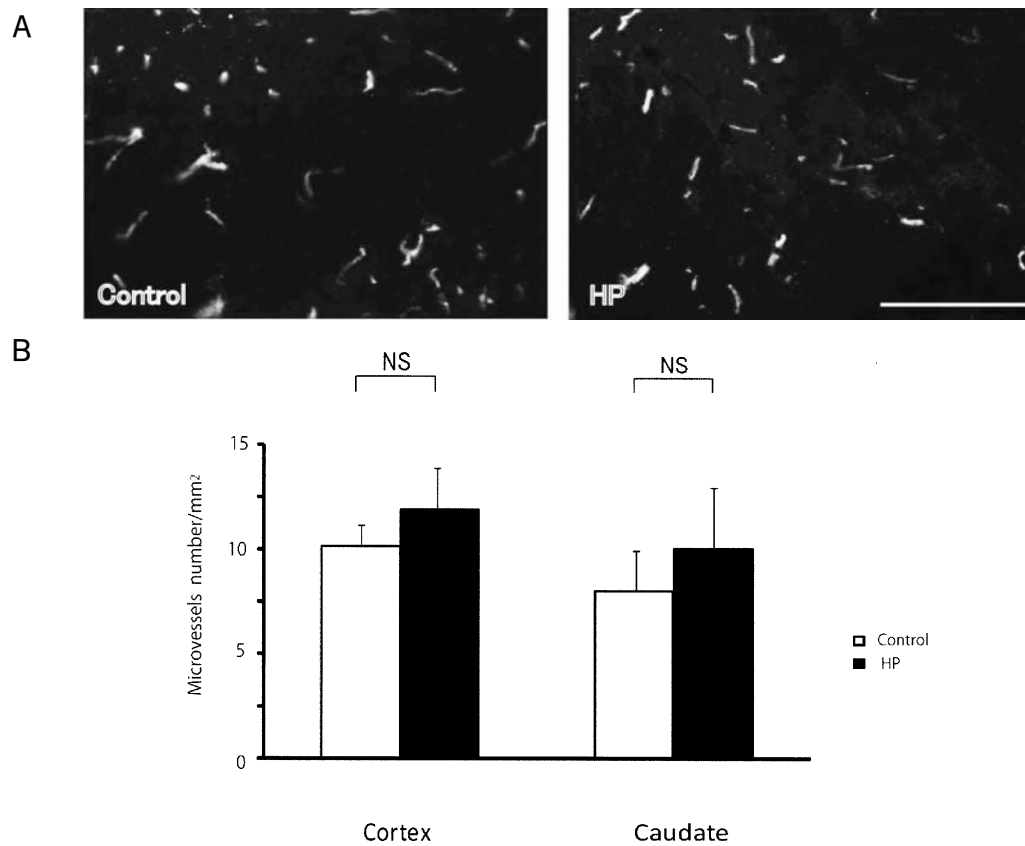
A

B

Cortex   Caudate

Fig. 5 A : FITC-labeled tomato lectin staining of microvessels in the cortex. Scale bar＝50 μm.
    B : Number of FITC-LEA-positive vessels contained in 1 mm² of cortex and caudate nucleus. Microvessel density in the HP group was not significantly increased compared with the control group.
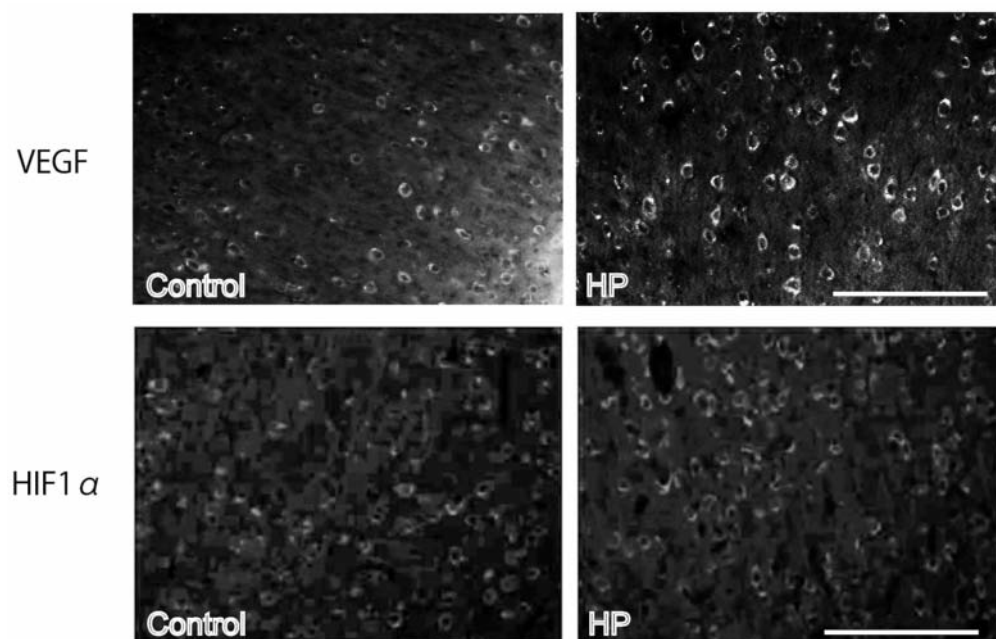
VEGF

HIF1 α

Fig. 6 Expression of VEGF and HIF-1α. Immunofluorescence staining for VEGF (green) and HIF-1α (red) in the cortex. Immunofluorescence signals of these proteins were observed predominantly in the cytoplasm of perikarya with morphology consistent with neurons, and fewer signals in microvessels and astrocytes. The fluorescence intensity of the HP group appeared stronger than that of the control group, although quantitative analysis was not performed. Representative results obtained from three individual experiments. Scale bar＝50 μm.

which is relevant to both clinical and experimental phenomena. The phenomenon of ischemic tolerance shown in this study might be related to complex mechanisms such as the reaction of endothelial cells against hypoxic stress resulting in a relative increase in the vessel density, although the true mechanism of the prolonged ischemic tolerance remains to be elucidated. Understanding ischemic tolerance may help to unravel the molecular and structural mechanisms involved in neuroprotection, and might improve therapeutic strategies for patients with stroke and other ischemia-related diseases.

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