Brain Structure and Function (2019) 224:727–738
https://doi.org/10.1007/s00429-018-1800-4

ORIGINAL ARTICLE

Preconditioning exercise reduces brain damage and neuronal apoptosis through enhanced endogenous 14-3-3γ after focal brain ischemia in rats

Shotaro Otsuka[1] · Harutoshi Sakakima[1] · Takuto Terashi[1] · Seiya Takada[1] · Kazuki Nakanishi[1] · Kiyoshi Kikuchi[2,3,4,5]

Received: 7 August 2018 / Accepted: 21 November 2018 / Published online: 26 November 2018

Abstract

14-3-3γ is an important early ischemia-inducible protective factor against ischemic cell death in cerebral cortical neurons. We investigated the anti-apoptosis mechanism of enhanced 14-3-3γ mediated by preconditioning exercise-induced brain ischemic tolerance after stroke. Rats were assigned to four groups: exercise and ischemia (Ex group), ischemia and no exercise (No-Ex group), exercise and no ischemia (Ex-only group), and no exercise and ischemia (control group). Rats were trained on a treadmill for 5 days a week for 3 weeks (running speed, 25 m/min; running duration, 30 min/day). After the exercise program, stroke was induced by left middle cerebral artery occlusion. The infarct volume, neurological deficits, and motor function, as well as expression levels of hypoxia-induced factor-1α (HIF-1α), 14-3-3γ, P2X7 receptors, p-β-catenin Ser37, Bax, and caspase 3 were evaluated by immunohistochemistry and western blotting. The expression of HIF-1α and 14-3-3γ significantly increased in neurons and astrocytes in the Ex-only group. HIF-1α was co-expressed with P2X7 receptor- and GFAP-positive astrocytes. After stroke, the Ex group had significantly reduced brain infarction. HIF-1α and 14-3-3γ significantly increased in the Ex group compared to the No-Ex group. In addition, p-β-catenin Ser37 significantly increased following elevated 14-3-3γ; in contrast, Bax and caspase 3 were significantly reduced in the Ex group. Our findings suggest that preconditioning exercise prior to ischemia induces neuron- and astrocyte-mediated brain ischemic tolerance through increased expression of HIF-1α and 14-3-3γ, which are intrinsic protective factors; the upregulated 14-3-3γ induced by preconditioning exercise reduces ischemic neuronal cell death through the 14-3-3γ/p-β-catenin Ser37/Bax/caspase 3 anti-apoptotic pathway.

Keywords 14-3-3γ · HIF-1α · Physical activity · Ischemic tolerance · Intrinsic factor

Introduction

Ischemic stroke is one of the leading causes of mortality and severe long-term morbidity worldwide (Prabhakaran et al. 2015). Preconditioning is an endogenous strategy that leads cells and organisms to initiate the expression of intrinsic protective factors, which help cells to acquire tolerance to and defend themselves against subsequent damage (Dong et al. 2010). Physical exercise may be a promising preconditioning method to induce brain ischemic tolerance through the promotion of angiogenesis, mediation of inflammatory responses, and inhibition of neuronal apoptosis (Zhang et al. 2011). Preconditioning exercise provides significant neuroprotection against acute stroke, reduces neural deficits, and ameliorates stroke-induced injury (Guo et al. 2008; Dornbos et al. 2013; Feng et al. 2014; Aboutaleb et al. 2015; Otsuka et al. 2016).

✉ Harutoshi Sakakima
sakaki@health.nop.kagoshima-u.ac.jp

✉ Kiyoshi Kikuchi
kikuchi_kiyoshi@kurume-u.ac.jp

[1] Course of Physical Therapy, School of Health Sciences, Faculty of Medicine, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8544, Japan

[2] Division of Brain Science, Department of Physiology, Kurume University School of Medicine, Kurume, Japan

[3] Department of Neurosurgery, Kurume University School of Medicine, Kurume, Japan

[4] Department of Systems Biology in Thromboregulation, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan

[5] Department of Pharmacology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Intrinsic protective factors include transcriptional factors and endogenous protective proteins. Hypoxia-induced factor-1α (HIF-1α) is one of the most important transcriptional factors implicated in the hypoxic or ischemic brain (Yang et al. 2018) and is rapidly upregulated in response to hypoxia. Accumulating evidence indicates that HIF-1α, a known mediator of ischemic tolerance, plays a key role in neuroprotection against ischemic brain injury (Ogle et al. 2012; Ryou et al. 2012), and HIF-1α mRNA is increased by preconditioning exercise (Dornbos et al. 2013). At the molecular level, HIF-1 binds to hypoxic elements and activates the transcription of many genes, which promote cell survival (Cao et al. 2014). Similarly, endogenous 14-3-3 proteins are important intrinsic protective factors to ameliorate ischemic damage and promote cell survival (Dong et al. 2010). 14-3-3γ is an isotype of the 14-3-3 protein family (β, η, γ, δ, τ, ζ, and ε) and is most abundant in the brain (Chen et al. 2003; Dong et al. 2010). HIF-1α and 14-3-3ζ are involved in the cascade of the progression of human glioma cells, indicating that tissue hypoxia and apoptosis are implicated in the progression of brain tumors (Cao et al. 2014). The γ member of the 14-3-3 family exerts pleiotropic effects during various physiological processes, such as cell proliferation and anti-apoptosis (Zhao et al. 2011; Pang et al. 2015). However, the relationship between 14-3-3γ and tissue hypoxia factors, such as HIF-1α, after preconditioning exercise remains unclear.

The mechanisms of ischemic tolerance in the central nervous system are complex and have not been fully elucidated. 14-3-3γ is an important early ischemia-inducible protective factor against ischemic cell death in cerebral cortical neurons (Lai et al. 2014). 14-3-3γ is expressed in both neurons and astrocytes with protective properties against ischemia insults (Chen et al. 2002; Dong et al. 2009), and it is upregulated in astrocytes following ischemic preconditioning in an in vitro study (Pang et al. 2015). 14-3-3γ exerts neuronal protection by suppressing p-β-catenin Ser37 and Bax functions in the cerebral cortical neurons during ischemia (Lai et al. 2014). β-Catenin is a critical transcriptional factor regulating cell death and survival (Krieghoff et al., 2006), and p-β-catenin Ser37 is distributed predominantly in the nucleus of cerebral cortical neurons upon ischemia (Zhao et al. 2005). Bax is a key ischemia-inducible cell death factor (Lai et al. 2014). However, the neuroprotective mechanisms of 14-3-3γ and the regulation of p-β-catenin Ser37 and Bax by preconditioning exercise-induced brain ischemic tolerance are not well understood. Therefore, we focused on the 14-3-3γ neuroprotective activity mediated by preconditioning exercise-induced ischemic tolerance after stroke.

In this study, we investigated the preconditioning exercise-induced brain ischemic tolerance by assessing the expression of HIF-1α and 14-3-3γ in cortical neurons and astrocytes. Furthermore, we examined the anti-apoptosis mechanisms of preconditioning exercise-induced 14-3-3γ after focal brain ischemia in rats.

Materials and methods

Animals

A total of 34 male Sprague-Dawley rats (mean body weight, 291.3 ± 38.0 g) were used in this study. The rats were pair-housed under temperature-controlled conditions (22.0 ± 1.0 °C) on a 12-h light/dark cycle, with food and water available ad libitum. The experimental protocol was approved by the Ethics Board of the Institute of Laboratory Animal Sciences of Kagoshima University.

Exercise training protocol

All rats were trained using a motor-driven treadmill (MK-680, MUROMACHI KIKAI CO., LTD, Japan) for 3 days (running speed, 15–25 m/min; running duration, 10 min/day) (familiarization). After familiarization, the rats were randomly divided into 4 groups: exercise and ischemia (Ex group, $n = 11$), ischemia and no exercise (No-Ex group, $n = 11$), exercise and no ischemia (Ex-only group, $n = 6$), and no exercise and ischemia (control group, $n = 6$). Before the middle cerebral artery occlusion (MCAO) procedure, the rats in the Ex and Ex-only groups exercised for 5 days per week (from Monday to Friday) for total 3 weeks (running speed, 25 m/min; running duration, 30 min/day). The rats in the No-Ex and control groups were allowed to move freely in their cages. The exercise was performed at 25 °C and during the daytime. Body weight was periodically measured to monitor the stress induced by treadmill running.

Middle cerebral artery occlusion (MCAO)

Rats were anesthetized using a combination of 0.3 mg/kg of medetomidine, 2.0 mg/kg of midazolam, and 2.5 mg/kg of butorphanol intraperitoneally 3 days after the end of treadmill running (3 weeks of running training). The rectal temperatures was monitored throughout the surgical procedure and maintained at 37 °C using a heating blanket (KN-474, NATSUME, Tokyo, Japan). Stroke was induced by a 60-min left MCAO using an intraluminal filament, as described previously (Sakakima et al. 2012; Otsuka et al. 2016). After 60 min of MCAO, reperfusion was established by withdrawal of the filament; 48 h later, the rats were killed. The brain, including the ischemic region, was analyzed histologically and immunohistochemically. Brain tissues were also used for 2,3,5-triphenyltetrazolium chloride (TTC) studies.

Evaluation of ischemic infarct

Rats were deeply anaesthetized with sodium pentobarbital and transcardially perfused with physiological saline before being decapitated. The brain was carefully removed and cut into seven 2-mm-thick coronal sections from the frontal tip using a brain slicer. The slices were then immersed in a 1% solution of TTC in phosphate buffered saline (PBS, pH 7.4) at 37 °C for 10 min. After staining, the sections were scanned to determine the ischemic infarct volume. The infarctions were measured using Scion Image software BETA 4.0.3 (Scion Corp, Frederick, MD). The total infarct area (mm^3) was multiplied by the thickness of the brain sections to obtain the infarct volume.

Evaluation of neurological scores, motor behavior, locomotor function, and sensorimotor function

Animals in each group were evaluated for neurological scores, sensorimotor dysfunction, and motor function using the beam walking test, rotarod task, and sticky tape-removal test 48 h after MCAO. After acclimatization with the beam walking and rotarod task, all animals were evaluated. A neurological grading system with a 5-point scale (0–4) was used. The scale was scored as follows: 0 = no apparent deficits; 1 = right forelimb flexion; 2 = decreased right forelimb grip while the tail was pulled; 3 = spontaneous movement in all directions; right circling only if pulled by the tail; 4 = spontaneous right circling.

In the motor behavior test, the rats were examined using a beam walking task with an elevated narrow beam (100 cm long × 2.5 cm wide). The time to traverse the beam was recorded and analyzed across three trials per day (maximum trial length, 60 s). The beam walking task was graded with a 6-point score (0–5). A score of 0 was given if the rat was unable to traverse the beam and could not place the affected limbs on the horizontal surface or maintain balance. A score of 1 was given if the rat was unable to traverse the beam or to place the affected limbs on the horizontal surface of the beam but was able to maintain balance. A score of 2 was given if the rat was unable to traverse the beam but was able to place the affected limbs on the horizontal surface of the beam and maintain balance. A score of 3 was given if the rat traversed the beam but used the affected limbs in less than half of its steps along the beam. A score of 4 was given if the rat traversed the beam and used the affected limbs to aid more than 50% of its steps along the beam. A score of 5 was given if the rat traversed the beam normally with no more than 2-foot slips. Before surgery, all animals underwent the motor behavior test to ensure that their performance scores were 5.

In the motor function and balance test, rats were examined using a rotarod task (MK-670, MUROMACHI KIKAI CO, LTD, Japan). Each rat was placed on the rotarod cylinder; the duration that the animal remained on the cylinder was measured. The rotation speed increased from 0 to 25 rpm in increments of 2.5 rpm every 6 s. The trial ended if the animal fell off the cylinder. Each animal was given three trials. The best latency to fall for each animal (in s) was used for analysis.

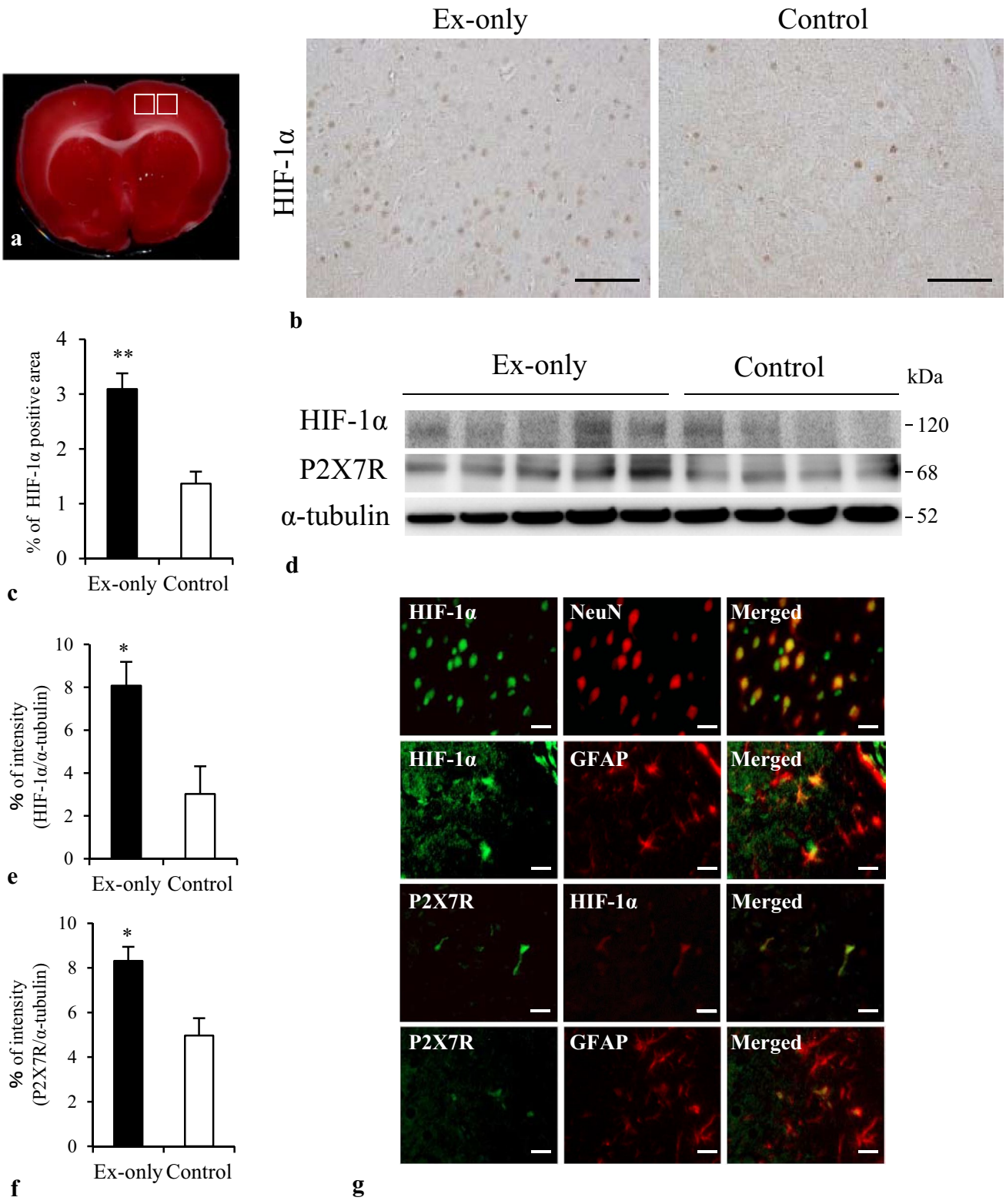
Sensorimotor dysfunction was assessed by the adhesive sticky tape removal test, as described previously (Otsuka et al. 2016). Square sticky labels (169 mm^2) were used as bilateral tactile stimuli occupying the palmar surface of each forepaw. The time to remove the label from the forelimbs was recorded in two trials for each forepaw. The better-recorded time in two trials was used for analysis. The maximum time allowed to remove the labels was 120 s.

Histology and immunohistochemistry

After TTC staining, brains were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight. After fixation, the tissue was processed for histology and immunohistochemistry. The paraffin-embedded coronal brain sections (4-μm thick) were stained with hematoxylin and eosin for the histological evaluation after MCAO.

The coronal brain sections were stained with the following antibodies: rabbit anti-HIF-1α antibody (Abcam plc, Cambridge, UK; ab216842), mouse anti-HIF-1α (Novus Biologicals, USA; NB100-131SS), rabbit anti-14-3-3γ antibody (ThermoFisher Scientific, USA; PA5-29690), mouse anti-glial fibrillary acidic protein (GFAP; a marker of activated astrocytes) antibody (Chemicon Int; MAB360), rabbit anti-P2RX7 (a maker of P2X7 receptor which is ATP acts as a ligand-gated ion channel) antibody (Abcam plc, Cambridge, UK; ab109054), mouse anti-neuronal nuclei (NeuN; a marker of neurons) antibody (Abcam plc, Cambridge, UK; ab104224), rabbit anti-activated caspase 3 (a marker of apoptotic activity) antibody (Santa Cruz CA, USA; sc-7148) or rabbit anti-activated caspase 3 (Proteintech, USA; 19677-1-Ap), and rabbit anti-Bax (a maker of pro-apoptotic protein) antibody (Abcam plc, Cambridge, UK; ab32503).

After deparaffinization and rehydration, the endogenous peroxidase was blocked with methanol containing 0.9% hydrogen peroxide for 15 min. The sections were rinsed with phosphate-buffered saline (PBS, pH 7.6) three times for 10 min each and were then blocked with 10% skim milk in PBS for 20 min. These sections were individually incubated at 4 °C overnight with the following antibodies: rabbit anti-HIF-1α antibody (1:100), rabbit anti-14-3-3γ antibody (1:3000), rabbit anti-Bax antibody (1:50), and rabbit anti-caspase 3 antibody (1:100). Subsequently, the sections were washed in PBS 3 times for 10 min each and then incubated with goat anti-rabbit IgG conjugated to a peroxidase-labeled dextran polymer (EnVision; Dako, CA, USA) for 60 min.



After the sections were rinsed with PBS, the immunoreactivity was visualized with a diaminobenzidine staining.

The co-localization of mouse anti-GFAP (1:1000) or mouse anti-NeuN (1:200) with rabbit anti-14-3-3γ (1:3000), rabbit anti-HIF-1α (1:100), rabbit anti-P2RX7 antibody (1:500), and rabbit anti-caspase 3 (1:100, Proteintech, USA, 19677-1-Ap) immunoreactivities were examined with immunofluorescence staining. In addition, the co-localization of

◂**Fig. 1** Preconditioning exercise enhanced the expression of HIF-1α in neurons and astrocytes. Representative TTC stained intact brain sections in the motor cortex (**a**), and two rectangle areas in the Ex-only and control groups (#3 out of 7 consecutive TTC sections from the cranial to caudate region) were used for immunohistochemical analysis. HIF-1α immunoreactivity significantly increased in the Ex-only group compared to that in the control group (**b, c**, $n=6$). Representative western blotting and semi-quantitative analysis showed that protein levels of HIF-1α and P2 X7 receptor (P2X7R) were significantly higher in the Ex-only group than in the control group (**d–f**, $n=5$ and 4, respectively). The HIF-1α positive cells were co-localized with the neuronal marker (NeuN) or astrocytic marker (GFAP) positive cells, and some P2 X7R positive cells were co-localized with the HIF-1α or GFAP positive cells in the Ex-only group (**g**). Date are presented as mean ± SE. $*p<0.05$, $**p<0.01$. Scale bar = 50 μm (**b**) or 20 μm (**g**)

mouse anti-HIF1α (1:100) with rabbit anti-P2RX7 antibody (1:500) immunoreactivities were examined with immunofluorescence staining. After incubation with two primary antibodies followed by washing with PBS, the sections were incubated with both Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:100) and Alexa Fluor 546-conjugated goat anti-mouse IgG antibody (1:100) for 60 min. After washing with PBS, sections were counterstained with 4′,6-diamino-2-phenylindole for 10 min and mounted with an aqueous mounting media. Immunofluorescent staining was observed with a fluorescence microscope (EVOS f1; AMG, Mill Creek, WA, USA).

Quantitative analysis of immunolabeled areas

Two areas in the motor cortex of the ischemic penumbra area of each immunostained section were imaged at 20× magnification using a microscope and with a camera without visual field overlap. The areas containing positive cells of HIF-1α, 14-3-3γ, activated caspase 3, and Bax were quantitatively measured in coronal sections (three out of the seven consecutive sections from the cranial to caudate region) using Scion Image software 4.0.3 (Scion Corp, Frederick, MD, USA). In addition, the motor cortex of the ischemic penumbra area of the caspase 3 and NeuN immunofluorescent staining section was imaged at 10× magnification using a fluorescence microscope and with a camera. The number of the NeuN positive neurons and survival neurons (NeuN positive cells which do not merge caspase 3) were counted in the motor cortex (0.74 mm^2) of the ischemic penumbra area. We calculated the ratios of the survival neurons (survival neurons/ total NeuN positive neurons). The quantitative analysis of each immunolabeled area was performed by two individuals, who were blind to the treatment group.

Western blotting

Immunoblotting was performed to detect protein expressions. HIF-1α, P2 X7 receptor, and 14-3-3γ were evaluated in the intact brain in the Ex-only ($n=5$) and control ($n=4$) rats. In addition, HIF-1α, 14-3-3γ, p-β-catenin Ser37, Bax, P2 X7 receptor, and caspase 3 were also examined in the ipsilateral brain, including ischemic regions, in the Ex and No-Ex rats ($n=4$ for each group). In brief, the cerebral cortex was dissected on ice and homogenized in T-Per reagent (Pierce Protein Research Products, 78510). Approximately 10 μg of protein in each sample was loaded in a 4–20% mini-protean precast gel (Bio-Rad, USA) and was subsequently transferred to a nitrocellulose membrane. After blocking with Tris-buffered saline/Tween 20 buffer (0.01 M TRIS–HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 3% skim milk for 1 h at 25 °C, the membrane was incubated with a primary antibody overnight at 4 °C and followed by a secondary horseradish peroxidase-labeled antibody. Detection was performed using the EzWestlumi plus detection system (ATTO, Tokyo, Japan). The following antibodies were used: rabbit anti-HIF-1α antibody (1:1200; Abcam plc, Cambridge, UK; ab216842); rabbit anti-14-3-3γ antibody (1:10,000; ThermoFisher Scientific, USA; PA5-29690); rabbit anti-P2RX7 antibody (1:1000; Abcam plc, Cambridge, UK; ab109054); rabbit anti-p-β-catenin Ser37 antibody (1:500; Bioss Inc, USA; bs-12854R); rabbit anti-Bax antibody (1:5000; Abcam plc, Cambridge, UK; ab32503); rabbit anti-caspase 3 antibody (1:1000; Santa Cruz CA, USA; sc-7148); and mouse anti-α-tubulin antibody (1:2000; Proteintech, USA; 66031-1g). Protein bands were visualized with chemical luminescence (WSE-6100 LuminoGraph I, ATTO, Tokyo Japan) and measured using Image J 1.46r software (National Institutes of Health). In addition, the α-tubulin was used as an internal loading control.

Statistical analysis

Statistical analyses were performed with parametric or non-parametric tests. Mann–Whitney *U* test was used to analyze the neurological and beam walking scores. An independent Student's *t* test was applied for between-group analysis. A *p* value of <0.05 was considered statistically significant. Data are expressed as mean ± standard error (SE). All data were analyzed using SPSS version 24 (IBM, Chicago, IL, USA).

Results

Preconditioning exercise enhanced the expressions of HIF-1α and 14-3-3γ in neurons and astrocytes in the cortex

We investigated whether intrinsic protective factors were upregulated by preconditioning exercise in the intact brain cortex (Fig. 1a). The immunoreactivity of HIF-1α

significantly increased by preconditioning exercise ($p < 0.05$; Fig. 1b, c), and the protein level of HIF-1α also significantly increased in the Ex-only group compared to the control group ($p < 0.05$; Fig. 1d, e). The HIF-1α positive cells were co-localized with NeuN (the neuronal marker) or GFAP (the astrocytic marker) positive cells (Fig. 1g). Additionally, because HIF-1α is involved in P2X7 receptor-mediated ischemic tolerance in astrocytes (Hirayama et al. 2015), we investigated the location of P2X7 receptor positive cells by immunofluorescence staining. The protein level of P2X7 receptor significantly increased in the Ex-only group compared to the control group ($p < 0.05$; Fig. 1d, f). Some P2X7 receptor positive cells were co-localized with the HIF-1α or GFAP positive cells in the Ex-only group, suggesting induction of ischemic tolerance in astrocytes by preconditioning exercise (Fig. 1g).

Similarly, the immunoreactivity of 14-3-3γ significantly increased by preconditioning exercise ($p < 0.05$; Fig. 2a, b), and the protein level of 14-3-3γ also significantly increased in the Ex-only group compared to the control group ($p < 0.05$; Fig. 2c, d). The 14-3-3γ positive cells were co-localized with NeuN or GFAP positive cells, however, it was largely observed in the NeuN positive cells (Fig. 2e).

Preconditioning exercise reduced the infarct volume, improved sensorimotor function, and attenuated neurological impairment after MCAO

A significant difference in infarct volumes was identified between the Ex (205.8 ± 50.9 mm^3) and the No-Ex (411.1 ± 57.0 mm^3) groups ($p < 0.05$; Fig. 3a, b).

Compared with those in the control group, the rats in the Ex and No-Ex groups showed significantly worse neurological and beam walking scores and increased sticky tape removal time after MCAO ($p < 0.01$). Walking time in the rotarod task significantly decreased in the No-Ex group (17.6 ± 4.4 s) than in the intact control group (51.3 ± 5.7 s); however, no significant differences were observed between the intact control and the Ex groups (41.7 ± 6.2 s). The sticky tape removal task was performed on both the left and the right (affected side) limbs. The remove latency was similar in the left

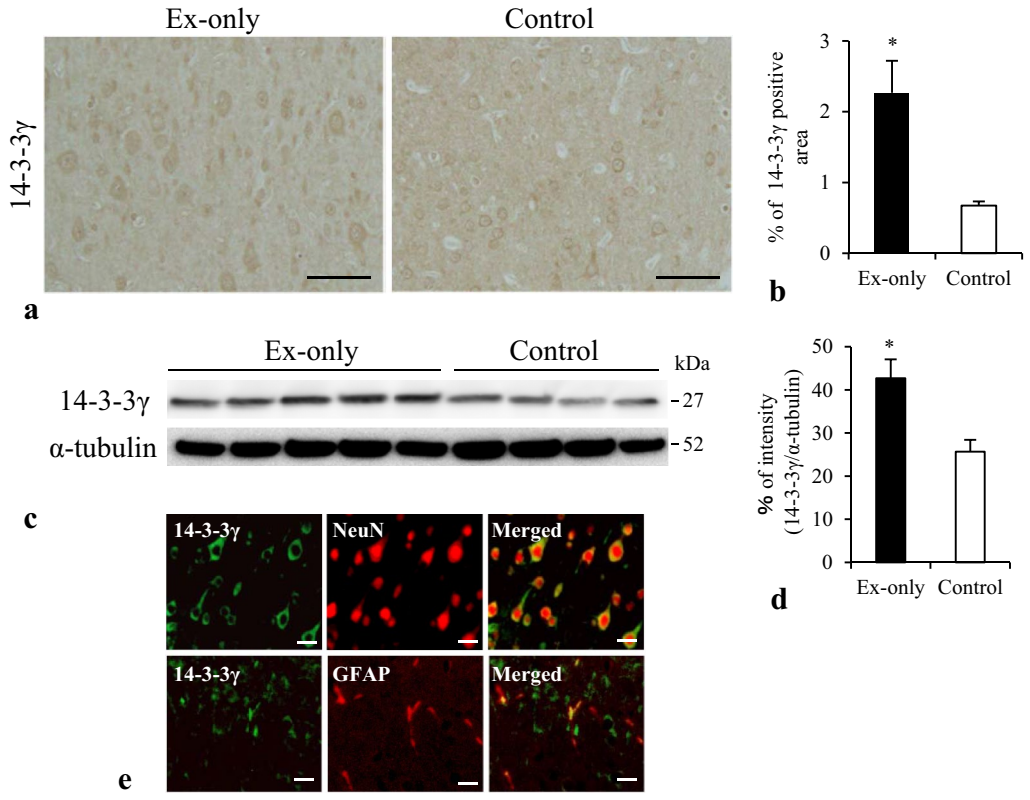


Fig. 2 Preconditioning exercise enhanced the expression of 14-3-3γ in neurons and astrocytes. 14-3-3γ immunoreactivity surrounding the lesion significantly increased in the Ex-only group than in the control group (**a, b**, $n = 6$). Representative western blotting and semi-quantitative analysis showed that protein levels of 14-3-3γ significantly increased in the Ex-only group than in the control group (**c, d**, $n = 5$ and 4, respectively). The 14-3-3γ positive cells were co-localized with NeuN or GFAP positive cells (**e**). Date are presented as mean ± SE. *$p < 0.05$. Scale bar = 50 μm (**a**) or 20 μm (**e**)

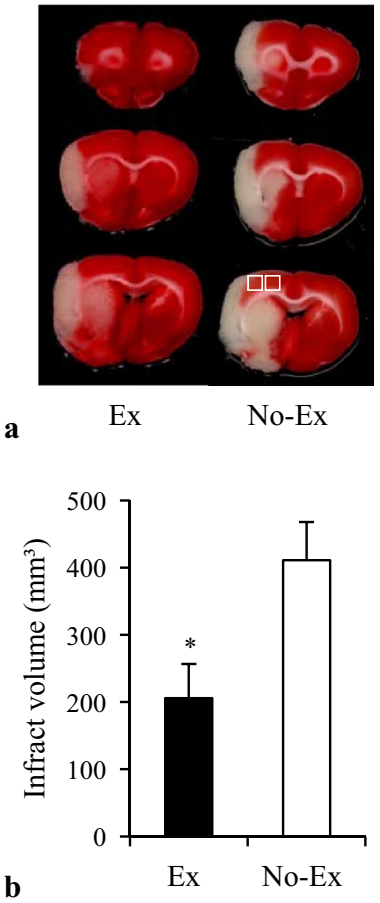


Fig. 3 Preconditioning exercise reduced the infarct volume at 48 h after MCAO. Representative TTC stained sections in the Ex and No-exercise groups (**a**), and two rectal areas (**a**) in the motor cortex of the ischemic penumbra (#3 out of 7 consecutive TTC sections from the cranial to the caudate region) were used for immunohistochemical analysis. The infraction volume significantly decreased in the Ex group than in the No-Ex group (**b**). Date are presented as mean ± SE ($n = 7$). *$p < 0.05$

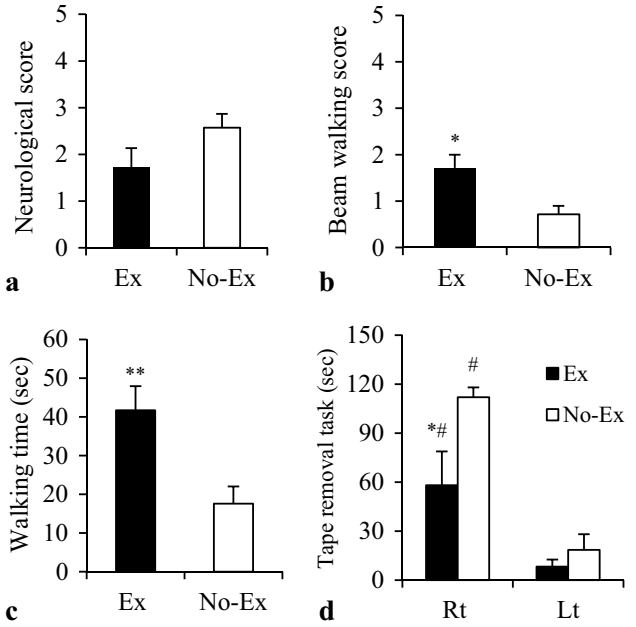


Fig. 4 Preconditioning exercise reduced sensorimotor function and neurological impairment after MCAO. The neurological scores (**a**), the beam walking scores (**b**), the rotarod walking time (**c**), and the sticky tape removal task (**d**) are shown in this Figure. The rats had significantly improved performance in the beam walking score, the sticky tape removal task, and rotarod walking time in the Ex group than in the No-Ex group. However, the neurological score was not significantly different between the Ex and the No-Ex groups. Date are presented as mean ± SE ($n = 7$). *$p < 0.05$, **$p < 0.01$ (compared with the No-Ex group). #$p < 0.05$ (compared with the left side)

and right limbs in the intact control group (12.5 ± 3.6 s, 11.9 ± 3.2 s, respectively).

Neurological deficits were ameliorated in the Ex group compared with the No-Ex group. However, no significant differences in the neurological scores were observed between the Ex and the No-Ex groups (Fig. 4a). Beam walking scores and walking times were significantly ameliorated in the Ex group compared to the No-Ex group ($p < 0.05$ or $p < 0.01$; Fig. 4b, c). The sticky tape removal task showed significantly longer latencies in the right limbs than in the left limbs in both the Ex and the No-Ex groups. However, the right limbs had significantly better latencies in the Ex group than in the No-Ex group ($p < 0.05$; Fig. 4d).

Preconditioning exercise increased the expressions of HIF-1α and 14-3-3γ in neurons and astrocytes after MCAO

After MCAO, the HIF-1α and 14-3-3γ immunoreactivity surrounding the lesions significantly increased in the Ex group compared to that in the No-Ex group after MCAO ($p < 0.05$; Fig. 5a–c). The HIF-1α and 14-3-3γ positive cells were co-localized with NeuN or GFAP positive cells (Fig. 5d, e). Similarly, the protein levels of HIF-1α and 14-3-3γ in the ipsilateral brain were significantly higher in the Ex group than in the No-Ex group ($p < 0.05$; Fig. 6a–c).

In addition, the P2X7 receptor positive cells were co-localized with GFAP positive cells in the Ex group (Fig. 5e). The protein levels of P2X7 receptor in the ipsilateral brain were significantly higher in the Ex group than in the No-Ex group ($p < 0.05$; Fig. 6a, d). These findings suggested that preconditioning exercise enhanced intrinsic protective factors in neurons and astrocytes after brain ischemia.

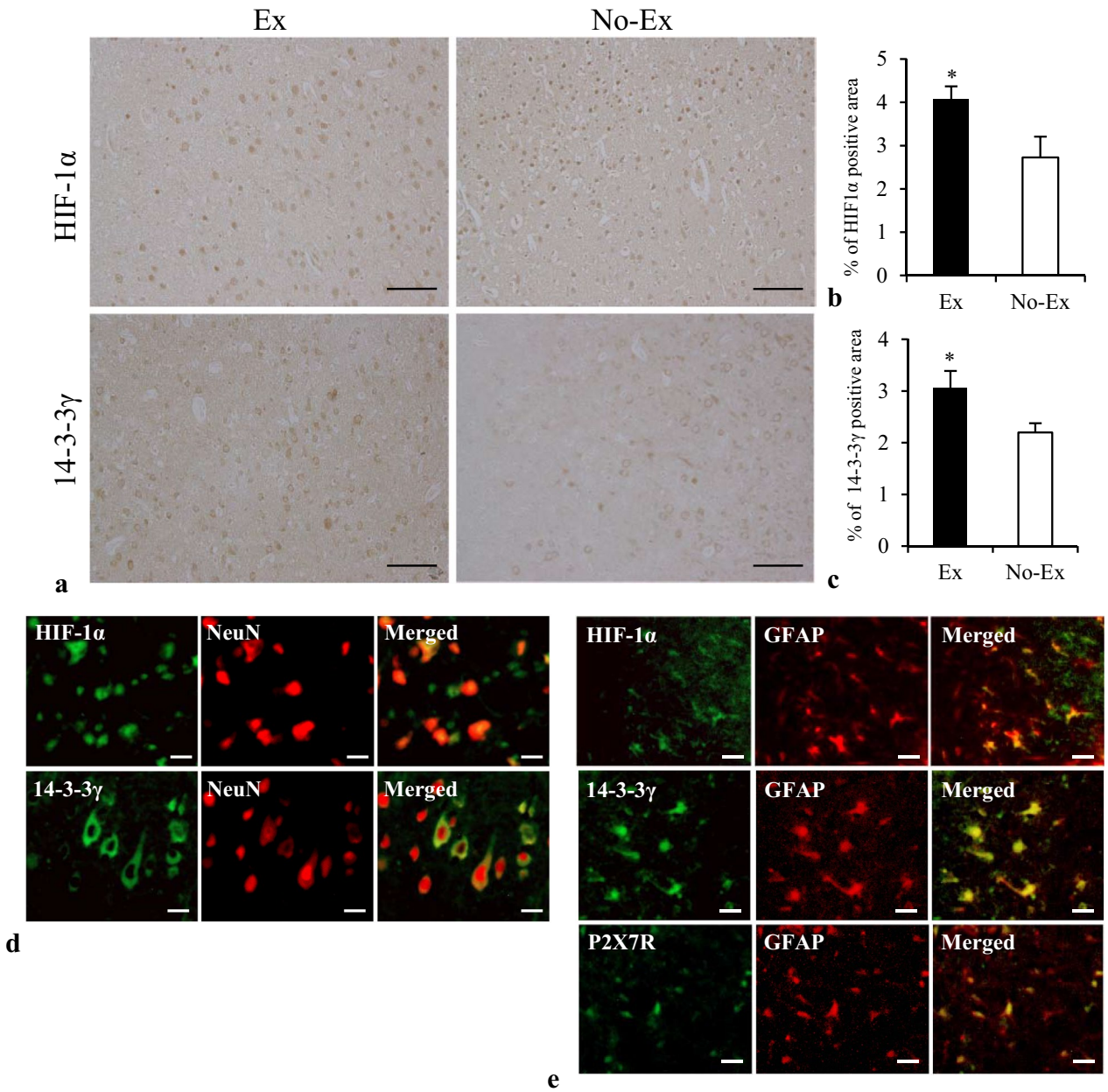


Fig. 5 The expression of HIF-1α and 14-3-3γ in neurons and astrocytes after MCAO. The left side of each photomicrograph (**a**) shows an ischemic region, and the right side shows a surrounding the lesion (penumbral area). HIF-1α and 14-3-3γ immunoreactivity surrounding the lesion significantly increased in the Ex group (**a**–**c**). The HIF-1α and 14-3-3γ positive cells were co-localized with NeuN or GFAP positive cells in the Ex group (**d, e**). The P2X7 receptor (P2X7R) positive cells were co-localized with GFAP positive cells in the Ex group (**e**). Date are presented as mean ± SE ($n = 7$). *$p < 0.05$. Scale bar = 50 μm (**a**) or 20 μm (**d, e**)

Preconditioning exercise reduced neuronal apoptosis via the 14-3-3γ/p-β-catenin Ser37/Bax/caspase 3 pathway after MCAO

The protein level of p-β-catenin Ser37 significantly increased in the Ex group compared to the No-Ex group after MCAO following increased14-3-3γ ($p < 0.05$; Fig. 6a, e). In contrast, the protein level of Bax, which is a pro-apoptotic protein, was significantly reduced in the Ex group compared to the No-Ex group after MCAO ($p < 0.05$; Fig. 6a, f). The caspase 3 protein levels, associated with the Bax protein levels, significantly decreased in the Ex group compared to those in the No-Ex group ($p < 0.05$; Fig. 6a, g). Similarly, the caspase 3 and Bax immunoreactivity surrounding the lesions were significantly reduced in the Ex group compared to that in the No-Ex group ($p < 0.05$; Fig. 7a–c). Caspase

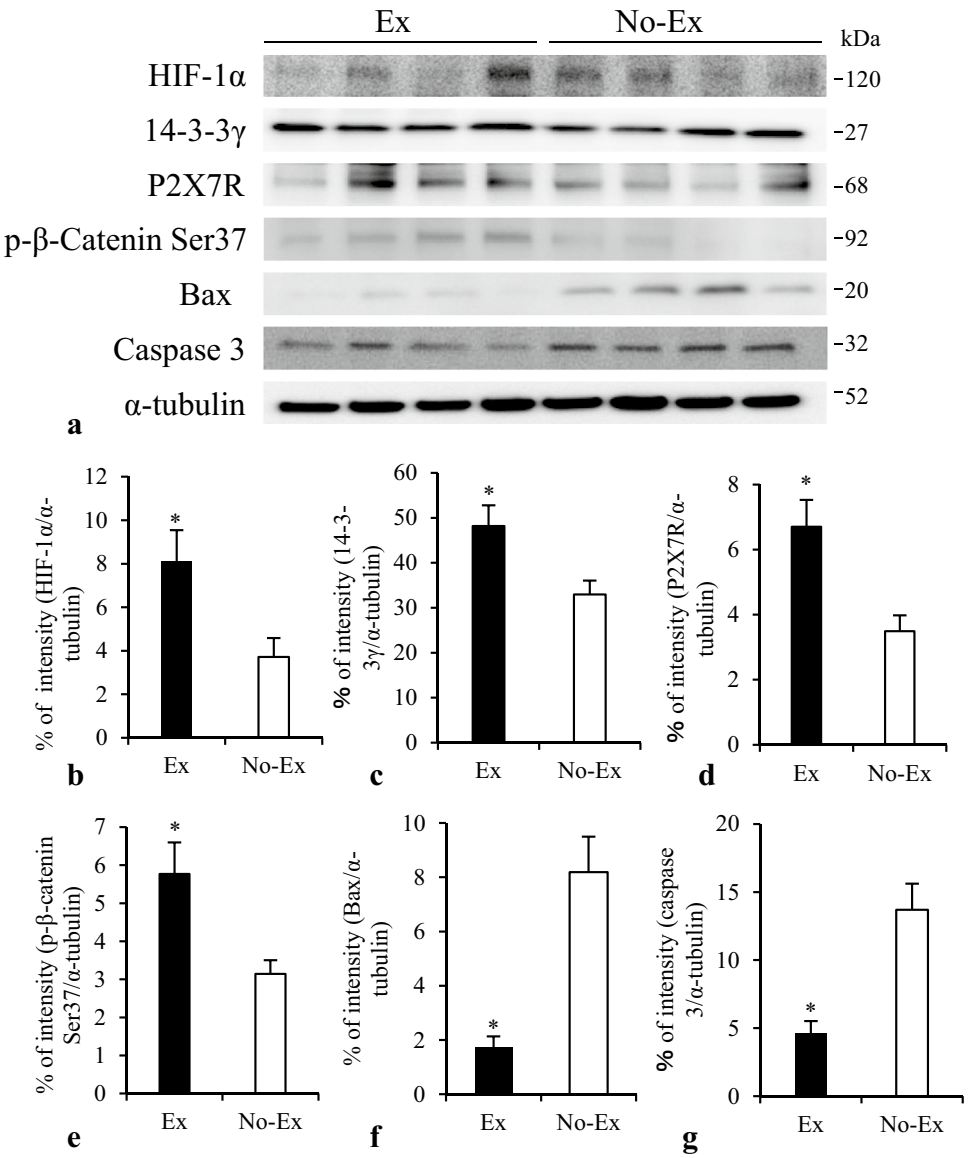


Fig. 6 Preconditioning exercise reduced neuronal apoptosis through the 14-3-3γ/p-β-catenin Ser37/Bax/caspase 3 pathway after MCAO. Representative western blotting and semi-quantitative analysis showed that protein levels of HIF-1α, 14-3-3γ, and P2X7 receptor (P2X7R) significantly increased in the Ex group than in the No-Ex group (**a**–**d**). The protein level of p-β-catenin Ser37 significantly increased in the Ex group following increased 14-3-3γ (**a, e**). In contrast, the protein levels of Bax and caspase 3 significantly decreased in the Ex group than in the No-Ex group (**a, f, g**). Date are presented as mean ± SE ($n = 4$). *$p < 0.05$

3 immunopositive cells were co-localized with the neuronal marker (NeuN) and astrocyte marker (GFAP), largely observed with the neuronal marker, indicating neuronal apoptosis surrounding the lesion (Fig. 7d). Therefore, we investigated the ratios of NeuN positive survival neurons surrounding the lesion area in the Ex and No-Ex groups. The ratios of survival neurons were significantly increased in the Ex group compared to that in the No-Ex group ($p < 0.05$; Fig. 7e).

Discussion

Many studies have demonstrated that various intrinsic factors have protective roles against ischemic insults. Numerous endogenous protective mechanisms are activated during the early phase of ischemic injury (Dong et al. 2010). In this study, we focused on neuroprotective mechanisms of HIF-1α and 14-3-3γ in the early phase after stroke. The results showed that HIF-1α and 14-3-3γ were activated in the neurons and astrocytes after preconditioning exercise, suggesting the presence of an ischemic tolerance in neurons and astrocytes. HIF-1α and 14-3-3 proteins are important intrinsic protective factors and play key roles in neuroprotection against ischemic brain injury (Dong et al. 2010; Yang et al. 2018). Our findings indicate that preconditioning exercise reduces the infarct volume, ameliorates sensorimotor function, and decreases neuronal apoptosis through enhanced expression of HIF-1α and 14-3-3γ and the activated 14-3-3γ/p-β-catenin Ser37/Bax/caspase 3 anti-apoptotic pathway, one of the underlying neuroprotective mechanisms, after ischemic stroke.

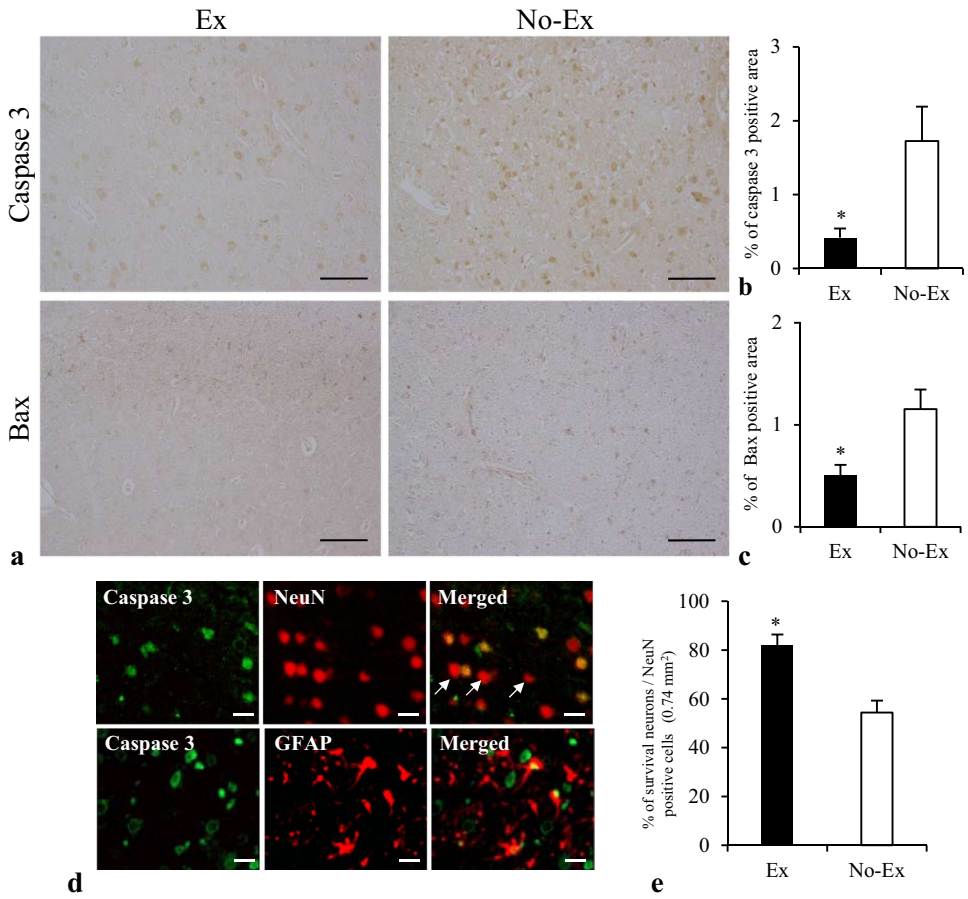


Fig. 7 The expression of caspase 3 and Bax surrounding the lesion after MCAO. The left side of each photomicrograph (**a**) shows an ischemic region, and the right side shows a surrounding the lesion (penumbral area). The caspase 3 and Bax immunoreactivities surrounding the lesion were significantly reduced in the Ex group (**a**–**c**). The caspase 3 immunopositive cells were co-localized with the neuronal marker (NeuN) and astrocyte marker (GFAP) in the Ex group (**d**), largely observed with the neuronal marker surrounding the lesion. The ratios of survival neurons (arrow) were significantly increased in the Ex group compared to that in the No-Ex group (**e**). Date are presented as mean ± SE ($n = 7$). *$p < 0.05$. Scale bar = 50 µm (**a**) or 20 µm (**d**)

The HIF-1α protein level increases following preconditioning exercise and plays key roles in neuroprotection against ischemic brain injury (Dornobs et al. 2013). HIF-1-regulated genes include vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase, and erythropoietin (Ogle et al. 2012). HIF-1 binds to hypoxic elements that activate the transcription of many genes, which promote angiogenesis and cell survival (Cao et al. 2014). Our previous study demonstrated that preconditioning exercise increased angiogenesis, enhanced expression of brain-derived neurotrophic factor (BDNF), and reduced neuronal apoptosis after stroke (Otsuka et al. 2016); these findings suggest that increased HIF-1α by preconditioning exercise is associated with angiogenesis or cell survival after stroke. In addition, HIF-1α is a known mediator of ischemic tolerance and is involved in P2X7 receptor-mediated ischemic tolerance in astrocytes (Hirayama et al. 2015). The P2X7 receptor play a dual role in cell survival and cell death depending on the intracellular pathways activated by the receptor (Monif et al. 2009). Therefore, in this study, we examined whether the P2X 7 receptor is a key molecule in astrocyte-mediated ischemic tolerance. Our results showed that HIF-1α was co-expressed with P2X7 receptor- and GFAP-positive astrocytes, suggesting that preconditioning exercise might induce astrocyte-mediated ischemic tolerance. Li et al. (2005) reported that 3-week treadmill running induced astroglial proliferation in the frontoparietal cortex in rats. However, our previous study showed that astrocyte proliferation did not occur in the rat intact cerebral cortex following preconditioning exercise (Otsuka et al. 2016). Hirayama et al. (2015) demonstrated that GFAP immunoreactivity increased at 3 and 6 days after preconditioning respectively in the striatum and cortex of rats, suggesting that the astrocyte activation pattern was different between the striatum and the cortex. Because we examined the brain at 3 days after preconditioning exercise, astrocyte proliferation might not significantly increase in the cortex following preconditioning exercise. However, preconditioning animals had significantly increased astrocyte proliferation surrounding the lesion in the cortex after stroke in our previous study (Otsuka et al. 2016). Astrocytes have enhanced expression of numerous protective proteins and factors, such as BDNF (Pang et al. 2015; Otsuka et al. 2016), and astrocytes could impart ischemic tolerance to neurons (Narayanan and Perez-Pinzon 2017). Astrocytes with ischemic tolerance induced by preconditioning exercise might be transformed qualitatively in the cerebral cortex. In fact,

astrocytes with ischemic tolerance induced by preconditioning exercise proliferated markedly during ischemic stroke, and increased astrocytes may have neuroprotective functions, through enhanced neurotrophic factors.

14-3-3γ is an early ischemia responsive gene and regulates cell death in the early stage of ischemic brain injury (Lai et al. 2014; Zhou et al. 2017). Our results showed that 14-3-3γ was upregulated by preconditioning exercise and protected against neuronal cell death after stroke. The 14-3-3γ expression is identified in neurons and astrocytes, and astrocytes may impart ischemic tolerance to neurons. In vitro study, 14-3-3γ is upregulated by ischemic preconditioning and protects astrocytes from ischemic injury (Pang et al. 2015). However, Chen et al. (2007) found that voluntary exercise decreased the protein level of 14-3-3γ in the hippocampus in aging rats. Our findings showed that both 14-3-3γ and HIF-1α were increased by preconditioning exercise. HIF-1α is implicated in the hypoxic or ischemic brain. The enhancement of 14-3-3γ may be associated with tissue hypoxia or ischemia. A positive correlation is found between 14-3-3ζ, HIF-1α, and VEGF in brain glioma (Cao et al. 2014). Therefore, preconditioning exercise may be associated with the expression of HIF-1α and 14-3-3 proteins.

14-3-3 proteins may be important in regulating other molecules to promote neuronal cell survival in vivo under stress conditions, including ischemia (Jang et al. 2009). 14-3-3γ is a selective ischemia-inducible survival factor in cerebral cortical neurons and reduces cell death by regulating the 14-3-3γ/p-β-catenin Ser37/Bax interaction in the nucleus (Lai et al. 2014). Our findings indicated that preconditioning exercise increased 14-3-3γ and p-β-catenin Ser37 and reduced Bax and caspase 3 after stroke, suggesting that upregulated HIF-1α and 14-3-3γ reduce ischemic neuronal cell death through the HIF-1α/14-3-3γ/p-β-catenin Ser37/Bax/caspase 3 anti-apoptotic pathway, one of the underlying neuroprotective mechanisms of preconditioning exercise.

A limitation of our study is that we did not examine other isotypes of 14-3-3 proteins. 14-3-3σ or 14-3-3η was upregulated in neurons or astrocytes in ischemic conditions, and 14-3-3ε and 14-3-3γ play a similar role in enhancing the sequestration of phosphorylated Bad and in suppressing apoptosis (Dong et al. 2010). In addition, no correlation has been assessed between the expression of HIF-1α, 14-3-3γ and p-β catenin Ser 37 after stroke, because of the small sample size. However, this study focused on endogenous neuroprotective mechanisms of 14-3-3γ by preconditioning exercise-induced brain ischemic tolerance in the early phase after stroke. The intrinsic protection induced by preconditioning exercise may be a promising target for the development of an alternative strategy of protection against ischemic damage.

Conclusion

Our findings indicate that preconditioning exercise induces ischemic tolerance through upregulation of HIF-1α and 14-3-3γ in the cortical neurons and astrocyte; preconditioning exercise reduces infarct volume and sensorimotor deficits after stroke. Additionally, our findings suggest that upregulated 14-3-3γ by preconditioning exercise reduce ischemic neuronal cell death via the 14-3-3γ/p-β-catenin Ser37/Bax/caspase 3 anti-apoptotic pathway, one of the underlying neuroprotective mechanisms after ischemic stroke.

Acknowledgements The authors greatly appreciate the assistance of Ms. Megumi Sumizono and Mr. Koki Ueda, and are grateful to Ms. Hui Gao for excrement technical assistance. This work was supported by grant from the JSPS KAKENHI (Grant no. JP17K01459 to Harutoshi Sakakima). This study was supported by grants from the JSPS KAKENHI (Grant no. JP16K10746 to Kiyoshi Kikuchi), General Insurance Association of Japan (to Kiyoshi Kikuchi), ZENKYOREN (National Mutual Insurance Federation of Agricultural Cooperatives) of Japan (to Kiyoshi Kikuchi), and Mitsui Sumitomo Insurance Welfare Foundation of Japan (to Kiyoshi Kikuchi).

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

Ethical approval All experiments were performed according to national and institutional guidelines and were approved by the appropriate authority.

References

Aboutaleb N, Shamsaei N, Khaksari M, Erfani S, Rajabi H, Nikbakht F (2015) Pre-ischemic exercise reduces apoptosis in hippocampal CA3 cells after cerebral ischemia by modulation of the Bax/Bcl-2 proteins ratio and prevention of caspase-3 activation. J Physiol Sci 65:435–443

Cao WD, Kawai N, Miyake K, Zhang X, Fei Z, Tamiya T (2014) Relationship of 14-3-3zeta (ζ), HIF-1α, and VEGF expression in human brain gliomas. Brain Tumor Pathol 31:1–10

Chen XQ, Yu ACH (2002) The association of 14-3-3γ and actin plays a role in cell division and apoptosis in astrocytes. Biochem Biophys Res Commun 296(3):657–663

Chen XQ, Chen JG, Zhang Y, Hsiao WW, Yu AC (2003) 14-3-3Gamma is upregulated by in vitro ischemia and binds to protein kinase Raf in primary cultures of astrocytes. Glia 42:315–324

Chen WQ, Viidik A, Skalicky M, Höger H, Lubec G (2007) Hippocampal signaling cascades are modulated in voluntary and treadmill exercise rats. Electrophoresis 28:4392–4400

Dong Y, Liu HD, Zhao R, Yang CZ, Chen XQ, Wang XH, Lau LT, Chen J, Yu AC (2009) Ischemia activates JNK/c-Jun/AP-1 pathway to up-regulate 14-3-3gamma in astrocyte. J Neurochem 109:182–188

Dong Y, Zhao R, Chen XQ, Yu AC (2010) 14-3-3gamma and neuroglobin are new intrinsic protective factors for cerebral ischemia. Mol Neurobiol 41:218–231

Dornbos D 3rd, Zwagerman N, Guo M, Ding JY, Peng C, Esmail F, Sikharam C, Geng X, Guthikonda M, Ding Y (2013) Preischemic exercise reduces brain damage by ameliorating metabolic disorder in ischemia/reperfusion injury. J Neurosci Res 91:818–827

Feng R, Zhang M, Wang X, Li WB, Ren SQ, Zhang F (2014) Preischemic exercise alleviates oxidative damage following ischemic stroke in rats. Exp Ther Med 8:1325–1329

Guo M, Cox B, Mahale S, Davis W, Carranza A, Hayes K, Sprague S, Jimenez D, Ding Y (2008) Pre-ischemic exercise reduces matrix metalloproteinase-9 expression and ameliorates blood–brain barrier dysfunction in stroke. Neuroscience 151:340–351

Hirayama Y, Ikeda-Matsuo Y, Notomi S, Enaida H, Kinouchi H, Koizumi S (2015) Astrocyte-mediated ischemic tolerance. J Neurosci 35:3794–3805

Jang SW, Liu X, Fu H, Rees H, Yepes M, Levey A, Ye K (2009) Interaction of Akt-phosphorylated SRPK2 with 14-3-3 mediates cell cycle and cell death in neurons. J Biol Chem 284:24512–24525

Lai XJ, Ye SQ, Zheng L, Li L, Liu QR, Yu SB, Pang Y, Jin S, Li Q, Yu AC, Chen XQ (2014) Selective 14-3-3γ induction quenches p-β-catenin Ser37/Bax-enhanced cell death in cerebral cortical neurons during ischemia. Cell Death Dis 5:e1184

Li J, Ding YH, Rafols JA, Lai Q, McAllister JP 2nd, Ding Y (2005) Increased astrocyte proliferation in rats after running exercise. Neurosci Lett 386:160–164

Monif M, Reid CA, Powell KL, Smart ML, Williams DA (2009) The P2×7 receptor drives microglial activation and proliferation: a trophic role for P2×7R pore. J Neurosci 29:3781–3791

Narayanan SV, Perez-Pinzon MA (2017) Ischemic preconditioning treatment of astrocytes transfers ischemic tolerance to neurons. Cond Med 1:2–8

Ogle ME, Gu X, Espinera AR, Wei L (2012) Inhibition of prolyl hydroxylases by dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires hypoxia inducible factor-1α. Neurobiol Dis 45:733–742

Otsuka S, Sakakima H, Sumizono M, Takada S, Terashi T, Yoshida Y (2016) The neuroprotective effects of preconditioning exercise on brain damage and neurotrophic factors after focal brain ischemia in rats. Behav Brain Res 303:9–18

Pang Y, Chai CR, Gao K, Jia XH, Kong JG, Chen XQ, Vatcher G, Chen JG, Vatcher G, Chen JG, Yu AC (2015) Ischemia preconditioning protects astrocytes from ischemic injury through 14-3-3γ. J Neurosci Res 93:1507–1518

Prabhakaran S, Ruff I, Bernstein RA (2015) Acute stroke intervention: a systematic review. JAMA 313:1451–1462

Ryou MG, Liu R, Ren M, Sun J, Mallet RT, Yang SH (2012) Pyruvate protects the brain against ischemia–reperfusion injury by activating the erythropoietin signaling pathway. Stroke 43:1101–1107

Sakakima H, Khan M, Dhammu TS, Shunmugavel A, Yoshida Y, Singh I, Singh AK (2012) Stimulation of functional recovery via the mechanisms of neuro repair by *S*-nitrosoglutathione and motor exercise in a rat model of transient cerebral ischemia and reperfusion. Restor Neurol Neurosci 30:383–396

Yang J, Liu C, Du X, Liu M, Ji X, Du H, Zhao H (2018) Hypoxia inducible factor 1α plays a key role in remote ischemic preconditioning against stroke by modulating inflammatory responses in rats. J Am Heart Assoc 7:e007589

Zhang F, Wu Y, Jia J (2011) Exercise preconditioning and brain ischemic tolerance. Neuroscience 177:170–176

Zhao H, Shimohata T, Wang JQ, Sun G, Schaal DW, Sapolsky RM, Steinberg GK (2005) Akt contributes to neuroprotection by hypothermia against cerebral ischemia in rats. J Neurosci 25:9794–9806

Zhao J, Meyerkord CL, Du Y, Khuri FR, Fu H (2011) 14-3-3 proteins as potential therapeutic targets. Semin Cell Dev Biol 22:705–712

Zhou XY, Hu DX, Chen RQ, Chen XQ, Dong WL, Yi CL (2017) 14-3-3 isoforms differentially regulate NFκB signaling in the brain after ischemia-reperfusion. Neurochem Res 42:2354–2362