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ORIGINAL PAPER

Honokiol suppresses the development of post-ischemic glucose intolerance and neuronal damage in mice

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Abstract Honokiol, a constituent of *Magnolia obovata*, has various pharmacological effects, including protection against cerebral ischemia. However, few studies have been conducted to evaluate the possible neuroprotective effects of honokiol against cerebral ischemia. We recently reported that cerebral ischemic neuronal damage could be triggered by glucose intolerance that develops after the onset of ischemic stress (i.e., post-ischemic glucose intolerance). In addition, suppression of post-ischemic glucose intolerance significantly ameliorated ischemic neuronal damage. Here, we investigated the effects of honokiol on the development of post-ischemic glucose intolerance and neuronal damage. Mice were subjected to middle cerebral artery occlusion (MCAO) for 2 h. The development of post-ischemic glucose intolerance on day 1 and neuronal damage on day 3 after MCAO were significantly reduced by intraperitoneal administration of honokiol (10 mg/kg) compared with the vehicle-treated group. Honokiol did not affect serum insulin or adiponectin levels. However, honokiol significantly decreased the expression of phosphoenolpyruvate carboxykinase and increased the expression of 5′-AMP-activated protein kinase (AMPK) on day 1 after MCAO, compared with the vehicle-treated MCAO group. The results of this study suggest that honokiol could prevent post-ischemic glucose intolerance in an AMPK-dependent manner, which may be involved in the neuroprotective effects of honokiol against cerebral ischemia.

Keywords Honokiol · Infarction · Glucose intolerance · Middle cerebral artery occlusion · 5′-AMP-activated protein kinase · Phosphoenolpyruvate carboxykinase

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Introduction

Self-medication is defined by the World Health Organization as the responsibility of individuals for their own health, and awareness that professional care for minor ailments is often unnecessary has contributed to this view [1]. From the point of view of self-medication, dietary supplements are widely preferred for the prevention of lifestyle-related diseases such as diabetes and hypertension [2]. Therefore, it is important to collect pharmacological evidence of the effects of nutrients or herbal supplements on such diseases.

Honokiol is a physiologically active, small-molecule polyphenol compound derived from *Magnolia obovata*. It is a traditional Chinese and Japanese medicine that is widely used to treat several diseases, including stroke and ischemic heart disease [3]. It was reported that honokiol has many pharmacological effects in the central nervous system including anti-oxidative, anti-inflammatory, anti-platelet, anti-anxiety, anti-depressant and anxiolytic effects [4–9]. Therefore, honokiol seems to exert a broad spectrum of effects with potential clinical relevance to brain ischemia, brain trauma and neurodegeneration.

In recent years, it has been reported that the administration of honokiol before and after induction of focal brain ischemia suppressed the development of infarction because of the anti-oxidative and anti-platelet effects of honokiol [10].

However, very few studies have evaluated the possible neuroprotective effects of honokiol against cerebral ischemia, and the mechanism underlying these effects is unclear.

Clinically, the severity of cerebral ischemic neuronal damage is often exacerbated in patients with a history of hyperglycemia and/or diabetes mellitus [11, 12]. This is at least partly explained by the finding that hyperglycemia can disrupt the blood–brain barrier, decrease cerebral blood flow, and impair cellular metabolism [13–17]. Accordingly, these events may be implicated in pre-ischemic hyperglycemia-induced aggravation of ischemic neuronal damage.

Recently, we found that cerebral ischemic stress per se causes hyperglycemia (i.e., post-ischemic glucose intolerance) and that normalization of blood glucose levels during the early phase of cerebral stroke decreased the extent of neuronal dysfunction [18]. These results suggest that the development of post-ischemic glucose intolerance could be a trigger of cerebral ischemic neuronal damage. In addition, our latest study showed that the serum adiponectin, an insulin-sensitizing adipokine, was significantly decreased in the early phase of cerebral ischemic stress [18]. These findings suggest that the signaling cascade underlying adiponectin receptors could be altered under ischemic stress. As one of the signaling molecules under adiponectin receptors, 5′-adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine kinase, is known to be activated by phosphorylation (pAMPK) and is known as a sensor of energy metabolism mainly expressed in liver [19, 20]. In peripheral tissues, AMPK is known to regulate glucose metabolism via suppression of gluconeogenesis [e.g., phosphoenolpyruvate carboxykinase (PEPCK)] in liver [21, 22], resulting in decrease of blood glucose levels. Honokiol was recently reported to stimulate glucose uptake in insulin-sensitive and insulin-resistant murine and human adipocytes by activating the insulin signaling pathway [23, 24]. Thus, we hypothesized that the protective effects of honokiol against cerebral ischemic stress are mediated by honokiol-induced improvements in insulin sensitivity. Here, we investigated this hypothesis and determined the effects of honokiol on the development of post-ischemic glucose intolerance and neuronal damage.

Materials and methods

Animals

Male ddY mice (5 weeks old) were obtained from SLC (Shizuoka, Japan). Animals were housed at a temperature of 23–24°C with a 12-h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. In addition, all experiments were approved by the ethics committee for animals at Kobe Gakuin University (approval number A060601-10).

Honokiol administration

Honokiol (Nacalai Tesque, Kyoto, Japan) was administered intraperitoneally at a dose of 1, 5, or 10 mg/kg in corn oil 15 min before inducing ischemia and 60 min after reperfusion [10]. The vehicle group received corn oil (0.1 mL/10 g) intraperitoneally.

Middle cerebral artery occlusion and reperfusion

Transient focal cerebral ischemia was established by performing middle cerebral artery occlusion (MCAO), as previously described [25]. In brief, mice were anesthetized with 2% isoflurane (Abbott Japan, Osaka, Japan) and maintained in an anesthetized state with 1% isoflurane. Then, the mouse was placed on an automatic heating pad (FH-100, Unique Medical, Osaka, Japan) connected to a sensor at the tip of a rectal thermometer (PTE-101, Unique Medical) and a small animal heat controller (ATC-101B, Unique Medical) to maintain body temperature at around 37 ± 0.5°C. The neck and carotid bifurcation were dissected. The left common carotid artery (CCA) was identified, and the external carotid artery and internal carotid artery (ICA) were used as a stump. A 8-0 nylon monofilament (Shirakawa, Fukushima, Japan) with a 4-mm tip coated in silicon resin (Provil® novo Medium BASE and Provil® novo Medium Catalyst, Heraeus Kulzer, Hanau, Germany) was introduced through the CCA via a small incision, and advanced 9 mm along the ICA beyond the bifurcation site. This stopped blood flow to the middle cerebral artery. After 2 h of ischemia, the mice were re-anesthetized with isoflurane and the filament was withdrawn to allow reperfusion. Sham-operated mice were subjected to the same procedure, but without MCAO. The operative site was sutured, and mice were allowed to awaken from the anesthesia. Mice with evidence of brain hemorrhage were removed from the study. Relative cerebral blood flow was measured by laser Doppler flowmetry (LDF; TBF-LN1, Unique Medical) to assess the extent of the vascular occlusion and reperfusion, as previously described [25].

Measurement of infarct volume

Mice were killed by cervical dislocation on day 3 after MCAO. The brains were cut into 2-mm-thick coronal slices (−2, 0, +2. and +4 mm from the bregma) using a brain slicer. The brain slices were then incubated in normal saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) for 10 min at 37°C. After

staining, brain slices were fixed with 4% paraformaldehyde (Sigma) for 2 h, and then stored in phosphate-buffered saline (PBS). Areas not stained red with TTC were considered to be infarctions. Brain slices were scanned and unstained areas (infarct areas) were measured by using Image J (NIH, Bethesda, MD, USA) and Adobe Photoshop Elements 5.0 (Adobe Systems Incorporated, Tokyo, Japan). The infarct volume (mm^3) was calculated by multiplying infarct volume (mm^3) and intensity (intensity = intensity of left hemisphere − intensity of right hemisphere) [26].

Neurological evaluation

The neurological status of the animals was assessed using the neurological deficit score (NDS) in which consciousness (0, normal; 1, restless; 2, lethargic; 3, stuporous; 4, seizures; and 5, death), walking (0, normal; 1, paw; 2, unbalanced walking; 3, circling; 4, unable to stand; and 5, no movement), limb tone (0, normal; 1, spastic; and 2, flaccid), and pain reflex was scored after reperfusion, as previously described [18, 25]. The pain reflex was assessed using the tail flick test (pain reflex = latency after MCAO − latency before MCAO). A cutoff time of 10 s was used to prevent tissue injury.

Learning and memory tests

A one-trial step-through-type passive avoidance learning test was used as previously described [18]. The apparatus (Ohara Co. Ltd., Tokyo, Japan) consisted of illuminated and dark compartments (each 4 × 13 × 10 cm) adjoining each other through a small gate (3 cm in diameter) with a grid floor consisting of 2.5-mm stainless steel rods set 7 mm apart. On the training trial (on day 2 after MCAO), MCAO-treated mice were placed in the illuminated compartment facing away from the dark compartment. When mice entered the dark compartment, an electric shock (50 V, 3 s duration) was delivered. Then, the mice were shut in the dark compartment for 5 s and carried back to the home cage. In the test trial, 24 h after the training trial (on day 3 after MCAO), the mice were again placed in the illuminated compartment, and the time taken for the mice to enter the dark compartment (maximum 600 s) was recorded.

Measurement of blood glucose and glucose tolerance

Mice were fasted for 15 h before the test day (on day 1 after MCAO), and 1.5 μL blood samples were obtained from the tail veins to measure blood glucose levels using the Glucose Pilot (Avenir Biotech, Carlsbad, CA, USA), as previously described [18]. Glucose tolerance was determined by oral glucose tolerance tests (OGTT). After mice had fasted for 15 h, the mice received an oral glucose load (2 g/kg body weight) and blood samples were obtained at 0, 30, 60, 120, and 180 min. The increments of blood glucose were calculated using the following formula: increment of fasting blood glucose (FBG) = FBG on day 1 after MCAO − FBG before MCAO; increment of blood glucose levels in OGTT = blood glucose level after administration of glucose − blood glucose level before administration. For the time-course experiments, we used 9–14 independent mice at each time.

Measurement of serum insulin and adiponectin levels

Serum insulin levels and adiponectin levels were measured in serum samples obtained at day 1 after MCAO using commercially available enzyme-linked immunosorbent assays (insulin ELISA kit, Morinaga Institute of Biological Science, Kanagawa, Japan; mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan), as previously described [18].

Measurement of homeostasis model assessment of insulin resistance index

The homeostasis model assessment of insulin resistance (HOMA-IR) index was used as an indicator of insulin sensitivity according to the following formula: [fasting insulin (μIU/mL) × fasting glucose (mg/dL)]/405.

Western blotting

Western blotting was performed as previously described [25, 27, 28] but with some modifications. Briefly, the liver was homogenized in homogenization buffer. Protein samples (30 μg) were electrophoresed on 10% SDS-PAGE acrylamide gels and then transferred onto nitrocellulose membranes (BioRad, CA, USA). AMPK and pAMPK were detected using primary antibodies purchased from Cell Signaling Technology (Beverly, MA, USA) and PEPCK was detected using antibodies from Santa Cruz (Santa Cruz, CA, USA) at dilutions of 1:1000. GAPDH was used as a loading control and was detected using primary antibodies from Chemicon (Temecula, CA, USA) at 1:20000. Blots for AMPK and pAMPK were incubated with the primary antibody overnight at 4°C in Tris-buffered saline containing 1% Tween-20 and 5% bovine serum albumin (Sigma). Blots for PEPCK and GAPDH were incubated in PBS containing 0.1% Tween-20 and blocking agent (GE Healthcare, Tokyo, Japan). After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000; KPL, Guildford, UK) for AMPK, pAMPK and PEPCK, or HRP-conjugated anti-mouse IgG (1:10000; KPL) for GAPDH for 1 h at room temperature. The immunoreactive bands were visualized using

Light-Capture (AE-6981; ATTO, Tokyo, Japan) with an ECL™ Western Blotting Analysis System (GE Healthcare). The signal intensity of immunoreactive bands was analyzed using a Cs-Analyzer (Ver. 3.0; ATTO).

Statistical analysis

Infarct volume, FBG, OGTT, serum insulin levels, HOMA-IR index, serum adiponectin levels and protein expression levels were compared by one-way analysis of variance (ANOVA) followed by Scheffe's test. Results are presented as mean ± standard error of the mean (SEM). NDS and results of the one-trial step-through passive avoidance test were compared using the Steel–Dwass test with post-hoc nonparametric multiple comparison tests. Data are presented as medians (25–75%). Differences were regarded as statistically significant at $p < 0.05$.

Results

Effect of honokiol on infarct volume, behavioral abnormality and memory disturbance after cerebral ischemic stress

Photographs of coronal brain sections after TTC staining confirmed the presence of infarcts in the cortex, hippocampus, and striatum on day 3 after MCAO in the vehicle-treated group (Fig. 1a). Honokiol significantly and dose-dependently suppressed the development of infarction compared with the vehicle-treated group (Fig. 1a, b). In addition, the development of behavioral abnormalities and memory disturbances were significantly and dose-dependently suppressed in the honokiol-treated group compared with the vehicle-treated group (Fig. 1c, d).

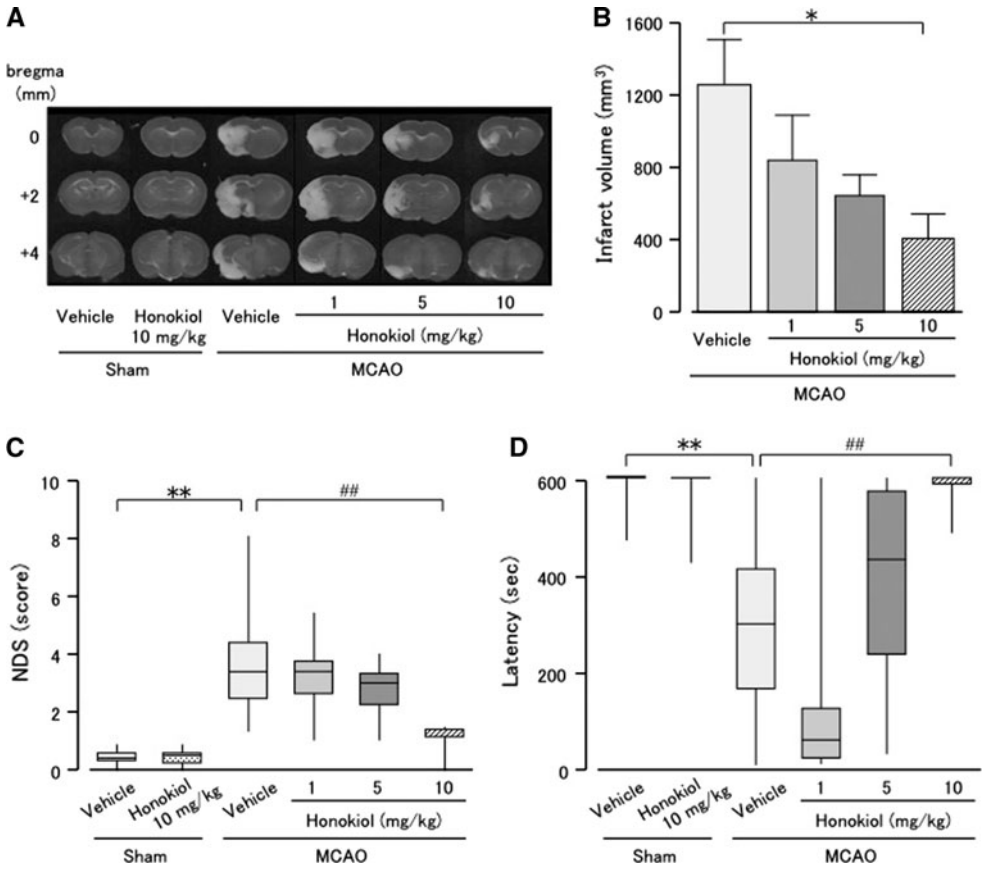

Fig. 1 Effect of honokiol on the infarct volume, behavioral abnormality and memory disturbance after cerebral ischemic stress. **a** Representative photographs of infarction at 0, +2, and +4 mm from the bregma in a coronal section stained with TTC on day 3 after MCAO. **b** Quantitative analysis of infarct volume. Results are presented as mean ± SEM. $^{\#}p < 0.05$, unpaired Student's t test. **c** Result of the NDS on day 3 after MCAO. **d** Result of the step-through-type passive avoidance learning test on day 3 after MCAO. Results of **c** and **d** are presented as median (25–75%). $^{\#\#}p < 0.01$, $^{**}p < 0.01$, Steel–Dwass test with post-hoc nonparametric multiple comparison tests. Vehicle-treated sham group: $n = 14$; honokiol-treated sham group: $n = 14$; vehicle-treated MCAO group: $n = 14$; honokiol (1 mg/kg)-treated MCAO group: $n = 8$; honokiol (5 mg/kg)-treated MCAO group: $n = 6$; honokiol (10 mg/kg)-treated MCAO group: $n = 11$

Effect of honokiol on transient increase in FBG after cerebral ischemic stress

There was a significant increase in FBG on day 1 after MCAO in the vehicle-treated group, which was dose-dependently and completely suppressed by honokiol (Fig. 2).

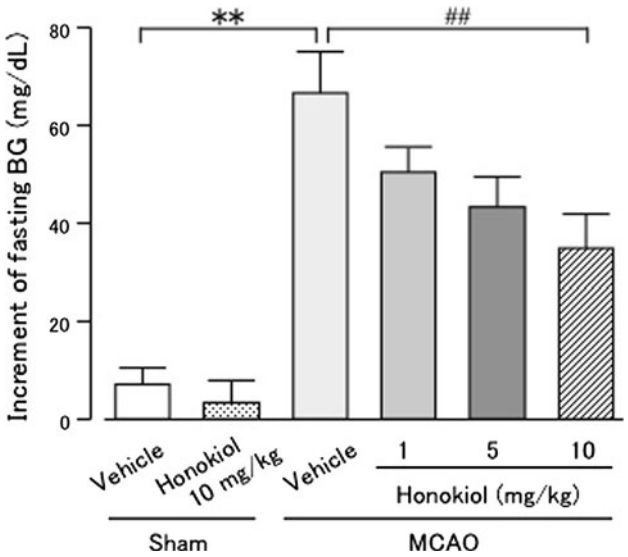

Fig. 2 Effect of honokiol on the transient increase in FBG after cerebral ischemic stress. FBG were measured on day 1 after MCAO. Results are presented as mean ± SEM. $^{**}p < 0.01$, $^{\#}p < 0.05$, one-way ANOVA and Scheffe's test. Vehicle-treated sham group: $n = 14$; honokiol-treated sham group: $n = 14$; vehicle-treated MCAO group: $n = 14$; honokiol (1 mg/kg)-treated MCAO group: $n = 8$; honokiol (5 mg/kg)-treated MCAO group: $n = 6$; honokiol (10 mg/kg)-treated MCAO group: $n = 11$

Effect of honokiol on development of glucose intolerance after cerebral ischemic stress

The increments in blood glucose levels during the OGTT on day 1 in the vehicle-treated MCAO group were significantly greater than those in the sham group (Fig. 3a). In contrast, honokiol significantly and dose-dependently reduced the increment in blood glucose levels during the OGTT compared with the vehicle-treated MCAO group (Fig. 3b).

Effect of honokiol on increase in serum insulin levels after cerebral ischemic stress

The serum insulin levels in the vehicle- and honokiol (10 mg/kg)-treated MCAO groups on day 1 were significantly higher than those in the sham group. Honokiol (10 mg/kg) did not affect the serum insulin levels compared with the vehicle-treated group (Fig. 4a).

Effect of honokiol on changes in HOMA-IR index after cerebral ischemic stress

The HOMA-IR indexes in the vehicle-treated MCAO groups on day 1 were significantly higher than those in the sham group. Honokiol (10 mg/kg) significantly suppressed the HOMA-IR index compared with the vehicle-treated group (Fig. 4b).

Effect of honokiol on decrease in adiponectin levels after cerebral ischemic stress

The serum adiponectin levels on day 1 in the vehicle- and honokiol (10 mg/kg)-treated MCAO groups were

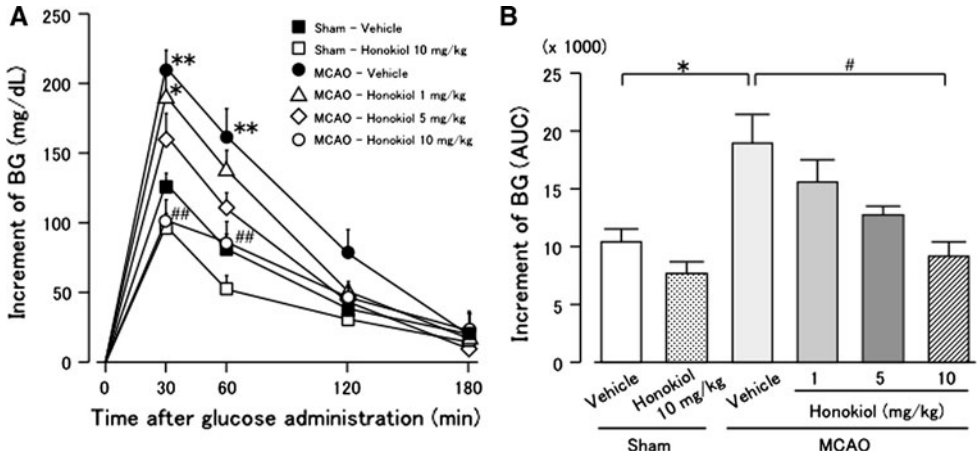

Fig. 3 Effect of honokiol on the development of glucose intolerance after cerebral ischemic stress. **a** Time course of oral glucose tolerance test (OGTT) on day 1 after MCAO. Mice were administered glucose (2 g/kg) orally. $^{**}p < 0.01$, $^{*}p < 0.05$, versus sham-vehicle, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, versus MCAO-vehicle. **b** Quantitative analysis of OGTT. Area under the curve of Fig. 3a was calculated. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{\#}p < 0.05$, one-way ANOVA and Scheffe's test. Vehicle-treated sham group: $n = 14$; honokiol-treated sham group: $n = 14$; vehicle-treated MCAO group: $n = 14$; honokiol (1 mg/kg)-treated MCAO group: $n = 8$; honokiol (5 mg/kg)-treated MCAO group: $n = 6$; honokiol (10 mg/kg)-treated MCAO group: $n = 11$

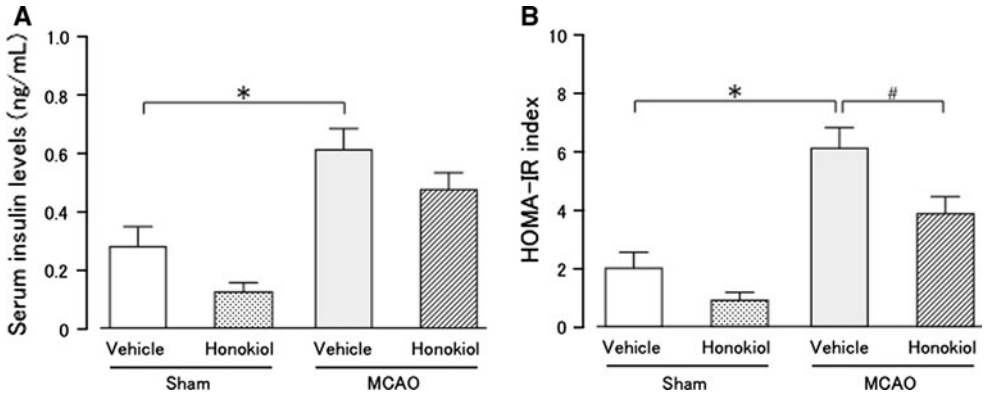

Fig. 4 Effect of honokiol on the increase in serum insulin levels and HOMA-IR index after cerebral ischemic stress. **a** Serum insulin levels were measured on day 1 after MCAO. Results are presented as mean ± SEM. **b** HOMA-IR index were measured on day 1 after MCAO. $^{*}p < 0.05$, $^{\#}p < 0.05$, one-way ANOVA and Scheffe's test. Vehicle-treated sham group: $n = 8$; honokiol-treated sham group: $n = 8$; vehicle-treated MCAO group: $n = 7$; honokiol-treated MCAO group: $n = 8$

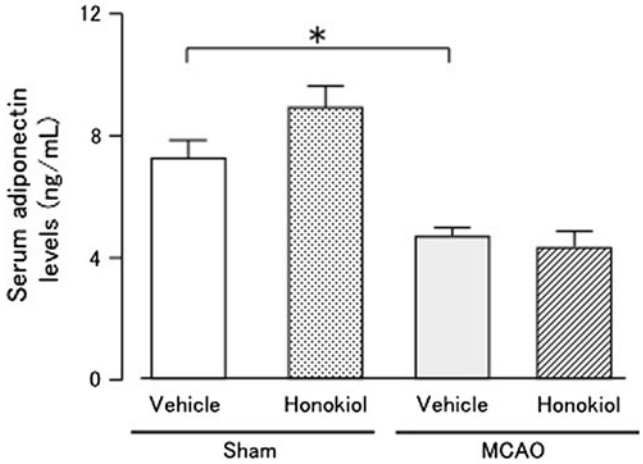

Fig. 5 Effect of honokiol on the decrease in adiponectin levels after cerebral ischemic stress. Serum adiponectin levels were measured on day 1 after MCAO. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{\#\#}p < 0.01$, one-way ANOVA and Scheffe's test. Vehicle-treated sham group: $n = 8$; honokiol-treated sham group: $n = 8$; vehicle-treated MCAO group: $n = 7$; honokiol-treated MCAO group: $n = 8$

significantly lower than those in the sham group. Honokiol (10 mg/kg) treatment did not affect the serum adiponectin levels compared with the vehicle-treated group (Fig. 5).

Effect of honokiol on hepatic PEPCK expression levels after cerebral ischemic stress

Hepatic PEPCK expression on day 1 was significantly greater in the MCAO group than in the sham group (Fig. 6). Honokiol (10 mg/kg) significantly suppressed the increase in hepatic PEPCK levels after MCAO (Fig. 6). In contrast, honokiol (10 mg/kg) did not affect PEPCK expression in the sham group.

Effect of honokiol on total AMPK and pAMPK in liver after cerebral ischemic stress

Hepatic pAMPK/AMPK expression on day 1 did not differ between the MCAO groups and the sham groups. However, honokiol (10 mg/kg) significantly increased hepatic pAMPK/AMPK expression in the MCAO group (Fig. 6).

Discussion

In this study, we investigated the mechanisms involved in the protective effects of honokiol against cerebral ischemic neuronal damage. In addition, we found that honokiol could attenuate post-ischemic glucose intolerance through activation of signaling molecules underlying insulin receptor and adiponectin receptor, including PEPCK and AMPK.

Reactive oxygen species are well established as playing a pivotal role in the pathogenesis of neuronal ischemic–reperfusion injury [29–31]. It has also been reported that many herbal medicines capable of suppressing the production of reactive oxygen species could prevent cerebral ischemic neuronal damage [18]. We have recently focused on the regulation of blood glucose levels as a new therapeutic strategy for cerebral ischemia. We previously reported that cerebral ischemic neuronal damage could be triggered by glucose intolerance developing after the onset of ischemic stress [18]. In addition, the suppression of post-ischemic glucose intolerance significantly ameliorated neuronal damage [18]. Interestingly, honokiol was reported to stimulate glucose uptake in adipocytes by activating components of the insulin signaling pathway [23, 24]. Therefore, it is possible that honokiol can regulate glucose metabolism. Accordingly, in this study, we focused on the effects of honokiol on the development of post-ischemic

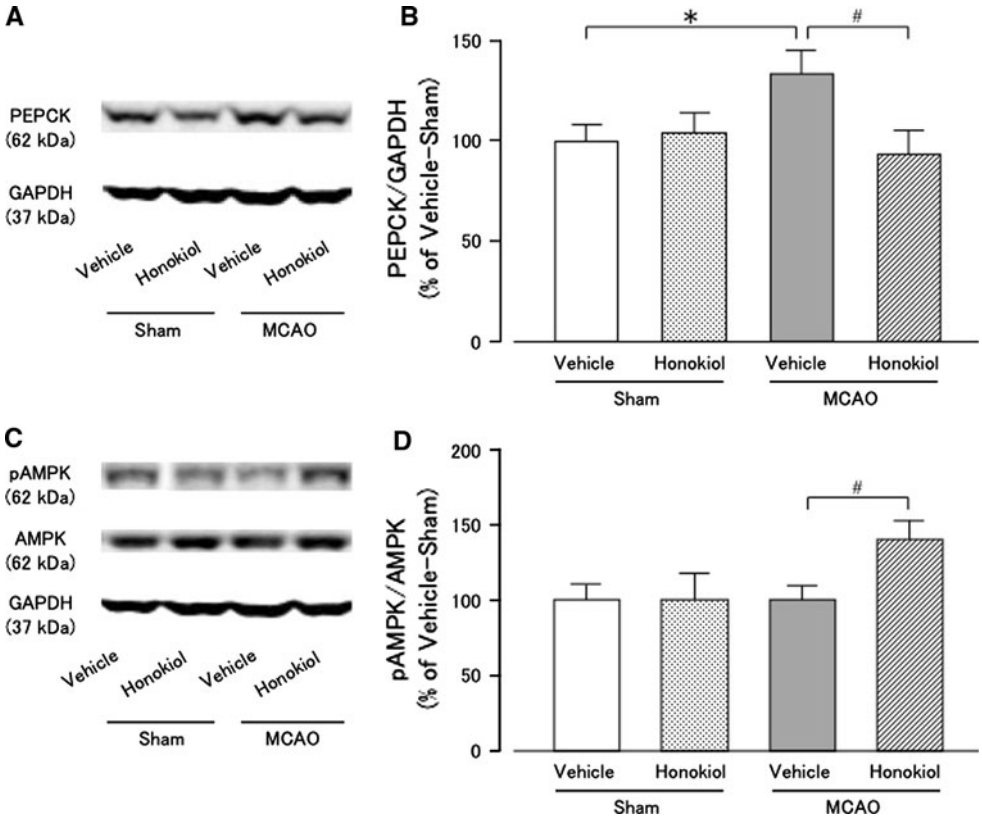

Fig. 6 Effect of honokiol on hepatic PEPCK, total AMPK and pAMPK expression levels after cerebral ischemic stress. **a** Representative photographs of Western blot analysis of hepatic pAMPK, AMPK and GAPDH. **b** The relative expression levels were analyzed by determining the ratio of pAMPK and AMPK (pAMPK/AMPK) at indicated periods. **c** Representative photographs of Western blot analysis of PEPCK and GAPDH levels. **d** Relative levels were analyzed by determining the ratio of PEPCK/GAPDH. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{\#}p < 0.05$, one-way ANOVA and Scheffe's test. Vehicle-treated sham group: $n = 8$; honokiol-treated sham group: $n = 8$; Vehicle-treated MCAO group: $n = 7$; honokiol-treated MCAO group: $n = 8$

glucose intolerance as a possible neuroprotective mechanism of honokiol. Marked post-ischemic glucose intolerance was observed on day 1 after MCAO, and was significantly reduced by honokiol. Therefore, the regulatory effects of honokiol on the development of post-ischemic glucose intolerance might be responsible for its protective effects against ischemic neuronal damage. Notably, honokiol did not affect post-ischemic glucose intolerance in the sham group, suggesting that these beneficial effects of honokiol are specific for the pathological state.

We found in this study that ischemic stress was associated with significantly increased serum insulin levels, which suggests that insulin secretory activity and/or β-cell glucose sensitivity in response to elevated blood glucose levels were enhanced in ischemic stress. Meanwhile, it was recently shown that adiponectin, an insulin-sensitizing adipokine secreted from adipose cells, can decrease hyperglycemia and reverse insulin resistance [32]. We previously reported that serum adiponectin levels were significantly decreased in the early phase of cerebral ischemic stress [18], suggesting that adipose cells experienced some damage during ischemic stress. Honokiol suppressed the development of post-ischemic glucose intolerance, but did not affect serum insulin or adiponectin levels after MCAO. On the other hand, the HOMA-IR index, a marker of insulin resistance, was improved by honokiol. These results indicate that the regulation of post-ischemic glucose intolerance as insulin resistance by honokiol was not due to enhanced insulin or adiponectin secretion. As described above, since honokiol improved insulin sensitivity, it is possible that honokiol activates several signaling molecules involved in the insulin signaling pathway in peripheral organs. In this study, we found that honokiol suppressed the ischemic stress-induced increase of PEPCK, a key gluconeogenic enzyme, and enhanced the activity of AMPK, a serine/threonine kinase that plays key roles in energy homeostasis and in the adiponectin signaling pathway [33]. Although the mechanism by which honokiol protects against post-ischemic glucose intolerance should be elucidated in more detail in future, it is possible that honokiol regulates glucose metabolism by activating AMPK and suppressing PEPCK during ischemic stress.

Honokiol has already been reported to exert pharmacological effects against ischemic stress through its anti-platelet [34], free radical scavenging [35], and antioxidant [36, 37] effects, and by suppressing inducible nitric oxide synthase expression [38]. The neuroprotective effects of honokiol may also involve suppression of post-ischemic glucose intolerance. These synergic effects of honokiol might explain its beneficial effects against cerebral ischemic stress-induced neuronal damage observed in this study.

In conclusion, we found that honokiol attenuates post-ischemic glucose intolerance, at least in part by regulating the expression of downstream components of the insulin and adiponectin signaling pathways. These effects of honokiol may mediate its protective effects against cerebral ischemic stress.

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