Effect of Orexin-A on Post-ischemic Glucose Intolerance and Neuronal Damage

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Abstract. Orexin-A is a newly identified neuropeptide expressed in the lateral areas of the hypothalamus that plays a role in various physiological functions, including regulation of glucose metabolism. We have previously reported that the development of post-ischemic glucose intolerance is one of the triggers of ischemic neuronal damage. Therefore, the aim of this study was to determine the effects of orexin-A on the development of post-ischemic glucose intolerance and ischemic neuronal damage. Male ddY mice were subjected to middle cerebral artery occlusion (MCAO) for 2 h. Neuronal damage was estimated by histological and behavioral analysis after MCAO. Intracerebroventricular administration of orexin-A (2.5, 25, or 250 pmol/mouse) significantly and dose-dependently suppressed the development of post-ischemic glucose intolerance on day 1 after MCAO and neuronal damage on day 3 after MCAO. In the liver and skeletal muscle, the expression levels of insulin receptor were decreased, whereas those of gluconeogenic enzymes were increased on day 1 after MCAO. Furthermore, these expressions were completely recovered to normal levels by orexin-A and were reversed by the administration of SB334867, a specific orexin-1 receptor antagonist. These results suggest that regulation of post-ischemic glucose intolerance by orexin-A suppressed cerebral ischemic neuronal damage.

Keywords: orexin-A, cerebral ischemia, glucose intolerance, insulin receptor, gluconeogenesis

Introduction

Stroke, including focal cerebral ischemic neuronal damage as a result of brain infarction, is the major cause of mortality, and the third most common cause of death worldwide (1, 2). In addition, the incidence and prevalence of stroke are expected to continue increasing (2). The development of stroke is related to lifestyle diseases, such as hypertension, dyslipidemia, and obesity (3). Most notably, diabetes mellitus and impaired glucose metabolism are the most important risk factors for stroke (4, 5). Clinically, cerebral ischemic neuronal damage is exacerbated in patients with a history of glucose intolerance or diabetes mellitus (6). We recently found that the cerebral ischemic stress per se causes hyperglycemia (i.e., post-ischemic glucose intolerance) and it may worsen ischemic neuronal damage in focal ischemic model mice (7, 8). In addition, decreased insulin sensitivity after cerebral ischemic stress seems to be involved in the development of post-ischemic glucose intolerance (7). Here, we focused on the changes in peripheral insulin receptor activity, specifically in the liver and skeletal muscle.

The orexin family (orexin-A and orexin-B) is a newly identified group of neuropeptides that are mainly expressed in the hypothalamus. Collectively, they play roles in many physiological functions, including arousal, energy homeostasis, glucose metabolism, feeding behavior, sleep, and wakefulness (9 – 12). Orexins are derived from a single precursor prepro-orexin and act via two types of G-protein-coupled receptor, the orexin-1 (OX1R) and orexin-2 (OX2R) receptors, which have a seven-transmembrane topology (10). OX1R has an affinity for orexin-A that is almost 50 times greater than that for orexin-B, while OX2R has comparable affinities for orexin-A and orexin-B (10). It was recently reported that the autonomic nervous system played an important role in conveying metabolic information between the hypothalamus and peripheral organs (13 – 16). Indeed, injec-
tion of orexin-A into the ventromedial hypothalamus of mice or rats enhanced insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle by activating the sympathetic nervous system (12). With regard to cerebral ischemia, the gene and protein expression of OX1R, but not OX2R, was reported to be increased in the ischemic hemisphere after focal ischemia in rats (17, 18), although the physiological significance of this remains unknown. Recently, orexin-A alters the pathological mechanisms involved in cerebral ischemic stress and has neuroprotective effects through improvement of cerebral blood flow after cerebral ischemia (19). In this study, we determined the effects of intracerebroventricularly (i.c.v.) administration of orexin-A on the development of post-ischemic glucose intolerance using focal cerebral ischemic model mice. Furthermore, we observed the effects of i.c.v. administration of orexin-A on peripheral insulin receptor signaling in liver and skeletal muscle. Finally, we investigated the effects of orexin-A on the development of ischemic neuronal damage.

Materials and Methods

Animals

The experiments were performed on male ddY mice (5-weeks-old, 25 – 30 g) obtained from SLC (Osaka). The animals were housed at a temperature of 23°C – 24°C with a 12-h light–dark cycle (lights on 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 ethical committee for animals at Kobe Gakuin University (approval number: A 060601-10).

Middle cerebral artery occlusion (MCAO) and reperfusion

The experimental transient focal ischemia mouse model was generated by performing MCAO, as previously described (20). Briefly, the left MCA was occluded for 2 h by inserting an 8-0 nylon monofilament with a thin silicon coat (Provil®novo Medium; Heraeus Kulzer, Hanau, Germany) through the common carotid artery followed by reperfusion while the mice were under isoflurane anesthesia. Sham-operated mice underwent the same surgical procedure without suture insertion. The relative cerebral blood flow was measured by laser Doppler flowmetry (LDF) (TBF-LN1; Unique Medical, Osaka) to assess the adequacy of the vascular occlusion and reperfusion while the mice were under isoflurane anesthesia. Sham-operated mice underwent the same surgical procedure without suture insertion. The relative cerebral blood flow was measured by laser Doppler flowmetry (LDF) (TBF-LN1; Unique Medical, Osaka) to assess the adequacy of the vascular occlusion and reperfusion, as previously described (20). Physiological parameters were measured before, during, and 30 min after MCAO using a sphygmomanometer (TK-370C; BrainScience Idea, Osaka) and i-STAT (300F; FUSO Pharmaceutical Industries, Osaka), as previously described (20).

Measurement of infarct volume

Mice were decapitated and the brains were immediately dissected. The brains were cut into 2-mm-thick coronal slices. The brain slices were incubated in saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) for 10 min at 37°C. The stained slices were then fixed with 4% paraformaldehyde (Sigma), and the infarct volumes were measured using image analysis software [Image J (NIH, Bethesda, MD, USA) and Adobe Photoshop Elements 5.0 (Adobe Systems Incorporated, Tokyo)], as previously described (20). The infarct volume was calculated based on the infarct area and intensity.

Neurological examination

Neurological examinations were performed after reperfusion using the neurological deficit score (NDS), which was comprised of 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 the pain reflex (20). The pain reflex was assessed using the tail flick test (pain reflex = latency after MCAO − latency before MCAO). A cut-off time of 10 s was used to prevent any injury to the tail.

Learning and memory tests

A one-trial step-through-type passive avoidance learning test was performed as previously described (7). Briefly, in the training trial, the mice were placed in the illuminated compartment facing away from the dark compartment. When the mice entered the dark compartment, an electric shock (50 V, 3 s in duration) was delivered. The mice were then returned to the home cage. In the test trial, 24 h after the training trial, the mice were placed in the illuminated compartment, and the latent time to enter the dark compartment (a maximum of 600 s) was measured.

Measurement of fasting blood glucose levels (FBG)

Starting 9 h after the onset of MCAO, the mice were fasted for 15 h to measure FBG, and blood samples (up to 1.5 μL) were obtained from the tail veins. Plasma FBG was measured using the Glucose Pilot (Aventir Biotech, Carlsbad, CA, USA). The increment in FBG was calculated using the formula: FBG increment = FBG after MCAO − FBG before MCAO, as previously described (7). For the time-course studies, we used 8 – 17 indepen-
dent mice at each time-point.

**Western blot analysis**

Western blotting was performed as previously described (8, 20) but with some modifications. Briefly, the liver and skeletal muscle were homogenized in homogenization buffer and protein samples (30 μg) were electrophoresed in 7.5% SDS-PAGE acrylamide gels and then transferred onto nitrocellulose membranes (BioRad, Hercules, CA, USA). Insulin receptor (InsR) and tyrosine-phosphorylated InsR (p-InsR) were detected using primary antibodies (1:1000) from Abcam (Tokyo). Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were detected using antibodies (1:1000 or 1:500) from Santa Cruz (Santa Cruz, CA, USA). Glycerinaldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control and was detected using primary antibodies (1:20000) from Chemicon (Temecula, CA, USA). Blots for InsR and p-InsR were incubated overnight with the primary antibody at 4°C in Tris-buffered saline containing 0.1% Tween-20 and 5% bovine serum albumin (Sigma), while PEPCK and G6Pase were incubated overnight in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and blocking agent (GE Healthcare, Tokyo). After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000; KPL, Guildford, UK) for InsR, p-InsR, and PEPCK; HRP-conjugated anti-mouse IgG (1:10000, KPL) for GAPDH; or HRP-conjugated anti-goat IgG (1:1000, KPL) for G6Pase for 1 h at room temperature. The immunoreactive bands were visualized using Light-Capture (AE-6981; ATTO, Tokyo) with an ECL™ Western Blotting Analysis System (GE Healthcare). The signal intensity of immunoreactive bands was analyzed by using a Cs-Analyzer (Ver. 3.0, ATTO).

**Measurement of serum insulin levels**

Serum insulin levels were measured using an insulin enzyme-linked immunosorbert assay kit (Morinaga Institute of Biological Science, Yokohama) on day 1 after MCAO (7).

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**Fig. 1.** Effects of orexin-A treatment on ischemic neuronal damage. Mice were treated with orexin-A (2.5, 25, and 250 pmol/mouse, i.e.v.) immediately after MCAO. A) Representative photographs of TTC staining on day 3 after MCAO. B) Quantitative analysis of the infarct volume. Results are presented as the mean ± S.E.M. *P < 0.05, †P < 0.05, n = 8 – 17. C and D) Results of the NDS and the step-through-type passive avoidance learning test on day 3 after MCAO. *P < 0.05, **P < 0.01, †P < 0.05, ‡P < 0.01, §P < 0.05, n = 8 – 17. SAL: saline. NDS: neurological deficit score.
Orexin-A administration
Mice were administered with 2.5, 25, or 250 pmol/mouse orexin-A, i.c.v. (Wako, Osaka) immediately after MCAO. SB334867, a selective non-peptide orexin-1 receptor antagonist (Tocris Bioscience, St. Louis, MO, USA), was dissolved in 1% dimethylsulfoxide and administered i.c.v. (2.5 nmol/mouse) 30 min before saline or orexin-A administration. All i.c.v. administrations were performed as previously described (21). Briefly, a microsyringe with a 27-gauge stainless-steel needle was used for all experiments. Tubing covered all but the terminal 2.5 – 3.0 mm of the needle to make a track through the brain and into the lateral ventricle but not through the floor of the lateral ventricle. The needle was inserted unilaterally into the lateral ventricle of the brain (1.0-mm lateral and 1.0-mm posterior to the bregma), as previously described (22). Correct needle position in the lateral cerebroventricle was verified by i.c.v. dye injection and subsequent post-mortem brain section verification of dye placement.

Statistical analyses
The infarct volume, FBG, and results of Western blots were analyzed by one-way analysis of variance followed by Scheffé’s test, and data are presented as means ± standard error of the mean (S.E.M.). NDS and one-trial step-through passive avoidance data were analyzed with a Steel-Dwass test with post-hoc nonparametric multiple comparison tests and data are presented as medians (25th – 75th percentile). A P-value of less than 0.05 was regarded as significant.

Results
Effects of orexin-A on ischemic neuronal damage
Orexin-A (2.5, 25, or 250 pmol/mouse) significantly and dose-dependently suppressed the development of infarction, neurological abnormalities, and memory disturbances on day 3 after MCAO, as compared with the saline-treated group (Fig. 1). These effects of orexin-A were significantly inhibited by SB334867, an OX1 antagonist (2.5 nmol/mouse) (Fig. 1).

Effects of orexin-A on the elevation of fasted blood glucose levels after cerebral ischemic stress
Orexin-A (2.5, 25, or 250 pmol/mouse) significantly and dose-dependently suppressed the elevation of FBG on day 1 after MCAO, as compared with the saline-treated group. However, the FBG in orexin-A–treated MCAO mice remained significantly higher than that in the sham group (Fig. 2). This effect of orexin-A was significantly inhibited by SB334867, an OX1 antagonist (2.5 nmol/mouse) (Fig. 2).

Fig. 2. Effects of orexin-A on the fasting blood glucose levels increment on day 1 after inducing ischemic stress. Mice were treated with orexin-A (2.5, 25, and 250 pmol/mouse, i.c.v.) immediately after MCAO. Results are presented as the mean ± S.E.M. *P < 0.05, **P < 0.01, †P < 0.05, n = 8 – 17. BG: blood glucose.

Fig. 3. Effects of orexin-A on changes in serum insulin levels caused by cerebral ischemic stress. Results are presented as the mean ± S.E.M. *P < 0.05, n = 8 – 16.
Effects of orexin-A on changes in the serum insulin levels after cerebral ischemic stress

The serum insulin levels were significantly increased on day 1 after MCAO. Orexin-A (250 pmol/mouse) did not affect the changes in serum insulin levels caused by MCAO (Fig. 3).

Effects of orexin-A on hepatic and skeletal muscle InsR and p-InsR expression after cerebral ischemic stress

The hepatic InsR and p-InsR protein expression levels were significantly lower in the MCAO group than in the sham group (Fig. 4) on day 1 after MCAO. On the other hand, in skeletal muscle, the InsR and p-InsR levels tended to be lower in the MCAO group, but not significantly, than in the sham group (Fig. 5). In the sham group, orexin-A significantly increased the hepatic or skeletal muscular InsR and p-InsR levels as compared with the saline-treated sham group (Figs. 4 and 5). Orexin-A (250 pmol/mouse) significantly suppressed the decrease in InsR and p-InsR expression in the liver and skeletal muscle on day 1 after MCAO (Figs. 4 and 5). These effects of orexin-A were significantly inhibited by

**Fig. 4.** Effects of orexin-A on changes in hepatic InsR (A) and p-InsR (B) expression on day 1 after inducing cerebral ischemic stress. Mice were treated with orexin-A (250 pmol/mouse, i.c.v.) immediately after MCAO. Representative Western blots of InsR, p-InsR, and GAPDH levels are shown. Relative levels were analyzed by determining the ratio of InsR/GAPDH and p-InsR/GAPDH. Results are presented as the mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.005, ###P < 0.001, ††P < 0.001, †††P < 0.001, $P < 0.05, n = 9 – 20.

InsR: insulin receptor. p-InsR: tyrosine-phosphorylated insulin receptor.
SB334867, an OXR1 antagonist (2.5 nmol/mouse) (Figs. 4 and 5).

**Effects of orexin-A on hepatic PEPCK and G6Pase expression after cerebral ischemic stress**

The hepatic PEPCK and G6Pase levels were significantly higher in the MCAO group than in the sham group (Fig. 6). In the sham group, orexin-A significantly decreased hepatic PEPCK and G6Pase protein expression, as compared with the saline-treated sham group (Fig. 6). In addition, orexin-A (250 pmol/mouse) significantly suppressed the increases in hepatic PEPCK and G6Pase protein expression in the MCAO group (Fig. 6). These effects of orexin-A were significantly inhibited by SB334867 (2.5 nmol/mouse) (Fig. 6).

**Discussion**

As we found in our previous reports (7, 8), ischemic neuronal damage could be triggered by glucose intolerance (post-ischemic glucose intolerance) that develops during the early phase of the onset of focal cerebral
Orexin-A Improves Ischemic Hyperglycemia

Ischemic stress. Here, we showed that cerebral ischemic stress significantly decreased the expression levels of InsR and p-InsR in the liver but not in skeletal muscle. Ischemic stress–induced increases in inflammatory cytokines, such as tumor necrosis factor (TNF)-α, may affect the transcription or degradation of the InsR (23). As previously reported, TNF-α down-regulates the insulin signaling through increasing protein tyrosine phosphatase 1B (23). It is well known that insulin signaling in the liver suppresses the transcription of gluconeogenic enzymes, particularly PEPCK and G6Pase (24, 25). Although serum insulin levels were increased by ischemic stress, it is possible that hepatic PEPCK and G6Pase were upregulated as a result of decreased InsR expression. In other words, ischemic stress caused the impairments in hepatic insulin sensitivity and this may be involved in the mechanism underlying post-ischemic glucose intolerance after MCAO.

Orexin-A, a neuropeptide produced in hypothalamus neurons, has been reported to be an important regulator of appetite and energy expenditure (26). In this study, we investigated the effects of orexin-A on hepatic PEPCK and G6Pase expression in a mouse model of cerebral ischemia. Figure 6 shows the effects of orexin-A treatment on hepatic PEPCK and G6Pase expression on day 1 after inducing cerebral ischemic stress. Mice were treated with orexin-A (250 pmol/mouse, i.c.v.) immediately after MCAO. Representative Western blots of PEPCK, G6Pase, and GAPDH levels are shown. Relative levels were analyzed by determining the ratio of PEPCK/GAPDH and G6Pase/GAPDH on day 1 after MCAO. Results are presented as the mean ± S.E.M. *P < 0.05, **P < 0.01, †P < 0.05, ‡P < 0.01, $P < 0.05, ††P < 0.05, n = 9–20. PEPCK: Phosphoenolpyruvate carboxykinase. G6Pase: glucose-6-phosphatase.

![Western blots showing effects of orexin-A treatment on hepatic PEPCK and G6Pase expression](image-url)
of glucose metabolism in peripheral organs via the activation of the autonomic nervous system (12). In this study, we found that exogenous orexin-A administered i.c.v. upregulated InsR and p-InsR expression in the liver and skeletal muscle. Orexin-A also decreased the expression of two hepatic gluconeogenic enzymes under ischemic stress, which suggests that orexin-A treatment may lead to the improvements of peripheral insulin sensitivity and in the suppression of post-ischemic glucose intolerance. As Shiuchi et al. reported that injection of orexin-A into the ventromedial hypothalamic nuclei (VMH) enhanced insulin-stimulated glucose uptake in skeletal muscle (12), the changes in glucose uptake activity in skeletal muscle may be involved in orexin-A–induced suppression of post-ischemic glucose intolerance.

Both OX1R and OX2R are widely expressed in the brain (26, 27). In the hypothalamus, the orexin receptors are highly expressed in the VMH, periventricular hypothalamic nucleus, arcuate nucleus (ARC), and lateral hypothalamic area (LHA) (26, 27) — origins of sympathetic or parasympathetic signals to the liver or skeletal muscle. It has been reported that activation of LHA and VMH neurons could regulate InsR substrate activity and glucose production in the liver and skeletal muscle via parasympathetic and sympathetic neurons, respectively (12, 28). I.c.v. administration of orexin-A may stimulate these pathways. Therefore, it seems likely that i.c.v. administration of orexin-A acted on the hypothalamic orexin receptor in this study.

Hypothalamic neuropeptides, such as orexin, neuropeptide Y (NPY), melanin-concentrating hormone, and proopiomelanocortin (POMC), are also involved in the regulation of feeding behavior and energy homeostasis via neuronal circuits in the hypothalamus (29 – 31). Orexins directly regulate these neuroendocrine systems in the ARC and VMH (29, 30). Hyperglycemia associated with peripheral insulin resistance is induced by lowering orexin levels and by lowering NPY and POMC expression in the ARC and VMH (29, 30). Hyperglycemia participates in subsequent neuronal damage. These findings provide some insight into the therapeutic effectiveness of the use of this endogenous neuropeptide.

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References

8 Harada S, Fujita-Hamabe W, Tokuyama S. The importance of regulation of blood glucose levels through activation of peripheral 5’-AMP-activated protein kinase on ischemic neuronal damage. Brain Res. 2010;1351:254–263.


