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Role of orexin in exercise-induced leptin sensitivity in the mediobasal hypothalamus of mice

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ABSTRACT

Orexin is known as an important neuropeptide in the regulation of energy metabolism. However, the role of orexin in exercise-induced leptin sensitivity in the hypothalamus has been unclear. In this study, we determined the effect of transient treadmill exercise on leptin sensitivity in the mediobasal hypothalamus (MBH) of mice and examined the role of orexin in post-exercise leptin sensitivity. Treadmill running for 45 min increased the orexin neuron activity in mice. Intraperitoneal injection of a submaximal dose of leptin after exercise stimulated the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in MBH of mice post-exercise compared with that in non-exercised mice, although intracerebroventricular (icv) injection of leptin did not enhance STAT3 phosphorylation, even after exercise. Icv injection of an orexin receptor antagonist, SB334867 reduced STAT3 phosphorylation, which was enhanced by icv injection of orexin but not by direct injection of orexin into MBH. Exercise increased the phosphorylation of extracellular signal-regulated kinases (ERKs) in the MBH of mice, while ERK phosphorylation was reduced by SB334867. Leptin injection after exercise increased the leptin level in MBH, whereas icv injection of SB334867 suppressed the increase in the leptin level in MBH of mice. These results indicate that the activation of orexin neurons by exercise may contribute to the enhancement of leptin sensitivity in MBH. This effect may be mediated by increased transportation of circulating leptin into MBH, with the involvement of ERK phosphorylation.

1. Introduction

The increasing number of obese people worldwide has created a major social problem. For example, for diabetic patients with obesity, medical expenses increase, and prognosis is poor [1]. Leptin is known to be secreted from adipose tissue, and it suppresses food intake and promotes energy metabolism via the hypothalamus [2]. However, obese patients show an increase in the blood leptin concentration and attenuation of the leptin effect [3,4]. This paradox is explained by leptin resistance. As a factor of this phenomenon, mechanisms such as defects in leptin transport in BBB, attenuation of phosphorylation of signal transducer and activator of transcription 3 (STAT3) in the MBH, loss and mutation of LEP or LEPR gene have been proposed [5]. Leptin resistance leads to further deterioration of obesity.

Chronic exercise improves leptin resistance as well as insulin resistance [6–9]. It has been reported that 4-week exercise training increased the expression of the leptin receptor, OB-Rb, in the ventromedial hypothalamus (VMH) [10], which is an important site in the MBH for leptin-induced glucose metabolism [11]. This finding confirms that exercise can be an effective treatment for metabolic diseases caused by leptin resistance. However, the effect of transient exercise on leptin sensitivity and on the improvement of leptin resistance in MBH is still unclear.

Orexin is a neuropeptide involved in pleiotropic functions such as regulation of arousal, muscle tone, and locomotor activity [12,13]. In addition, orexin is known to play an important role in the regulation of energy metabolism. Orexin neuron deficiency causes

Abbreviations: BBB, blood–brain barrier; ERK, extracellular signal-regulated kinase; icv, intracerebroventricular(ly); ip, intraperitoneal(ly); MBH, mediobasal hypothalamus; VMH, ventromedial hypothalamus.

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not only narcolepsy and a decrease in food intake but also obesity in mice [14]. In addition, there is also the knowledge that orexin drives spontaneous activity [15]. Narcoleptic patients show an increase in the body weight [16]. We have previously reported that orexin regulates the skeletal muscle glucose metabolism via VMH [17], which abundantly expresses the orexin type 1 receptor [18]. Since exercise generally elevates the arousal level, we hypothesized that exercise-activated orexin neurons and secreted orexin would synergistically enhance leptin sensitivity in MBH.

In this study, we determined the effect of transient treadmill exercise on leptin sensitivity in MBH of mice and evaluated the role of orexin in post-exercise leptin sensitivity in MBH using an icv injection of an orexin receptor antagonist, SB334867.

2. Materials and methods

2.1. Animals

We used 16- to 24-week-old C57BL/6J male mice (Japan SLC, Shizuoka, Japan). All mice were housed individually at a constant room temperature of 23 ± 1 °C with a 12-h light/dark cycle (lights on at 8 a.m.) and were fed a standard non-purified diet (Oriental Yeast, Tokyo, Japan) with food and water available ad libitum. All experiments were approved by the Tokushima University Animal Study Committee and conducted in accordance with the Guidelines for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

2.2. Treadmill running

Mice performed a transient treadmill running exercise (10 m/min, 45 min) after habituation to treadmill running for 3 days before the experiment. Only the mice that completed the endurance running were used in this experiment.

2.3. Surgical operation on animals

Mice were anesthetized by intraperitoneal (ip) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and a double-walled stainless steel cannula (Plastics One, Roanoke, VA, USA) was implanted stereotaxically into bilateral MBH, whereas a stainless steel cannula was implanted unilaterally into the lateral ventricle of the mice according to an atlas [19]. The stereotaxic coordinates for MBH were AP 1.5 (1.5 mm anterior to the bregma), L 0.3 (0.3 mm lateral to the bregma), and H 5.8 (5.8 mm below the bregma). The stereotaxic coordinates for lateral ventricle were AP 0.3, L 1.0, and H 2.4. The cannulas were anchored firmly to the skull with acrylic dental cement.

2.4. Administration of orexin, SB334867 and leptin

Orexin-A (Peptide Institute, Osaka, Japan), dissolved in physiological saline, was injected using a Hamilton microsyringe into unrestrained mice through the unilateral cannula implanted into the lateral ventricle (1 μL) or MBH (0.2 μL). The orexin type 1 receptor antagonist SB334867 (10 mM) (Tocris Cookson, Bristol, UK) was injected (1 μL) into the lateral ventricle. Mice received ip (2 mg/kg) or icv (1 μg) injection of leptin after running.

2.5. Western blotting

Forty-five minutes after leptin injection, MBHs were rapidly isolated from cervically dislocated mice and stored at −80 °C until analysis. The tissues were homogenized with cold lysis buffer, pH 7.5, containing protease inhibitors (P8370, Sigma–Aldrich) and phosphatase inhibitors (P5726, Sigma–Aldrich). Protein was denatured by boiling in the loading buffer. Twenty micrograms of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h and then incubated with rabbit antibodies (1:1,000; Cell Signaling Technology, Dallas, TX, USA) against phospho-STAT3 (Tyr-705) and STAT3. The blots were visualized with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:2,000; Santa Cruz Biotechnology, Camarillo, CA, USA) and an Immobilon western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA). The ImageJ software was used for the analysis.

2.6. Immunohistochemistry

This was following fixation in 4% paraformaldehyde in 0.1 M phosphate buffer before the brains were excised. The brains were removed and post-fixed for 24 h. The fixed brain was incubated at 4 °C for 2 days in phosphate-buffered saline (PBS) containing 20% (w/v) sucrose. The tissue was then embedded in OCT compound (Sakura FineTechnical, Tokyo, Japan), immediately frozen, and stored at −80 °C until analysis. Serial 30-μm cryosections were prepared using a cryostat (CM1850; Leica, Wetzlar, Germany), with the brain oriented for sectioning according to the mouse stereotaxic atlas [19]. Three sections corresponding to the lateral hypothalamus (between −1.46 and −2.06 mm posterior to the bregma) or medial basal hypothalamus (between −1.46 and −1.70 mm posterior to the bregma) or median eminence (between −1.70 and −1.94 mm posterior to the bregma) were selected per mouse.

For double immunohistofluorescence analysis of c-Fos and orexin, brain sections were exposed to 3% normal donkey serum for 2 h at room temperature and then incubated for 3 days at 4 °C with goat antibodies against orexin (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit antibodies against cFos (1:1,000 dilution; Cell Signaling Technology (CST), Danvers, MA, USA). After washing the sections with PBS, immune complexes were detected by incubation for 2 h at room temperature with an Alexa Fluor-594-labeled donkey anti-goat IgG antibody (Molecular Probes, Eugene, OR, USA) at a dilution of 1:500 or with an Alexa Fluor-488-labeled donkey anti-rabbit IgG antibody (1:500; Molecular Probes). The sections were finally examined under a microscope (DM4000B; Leica) and carefully matched based on the shape of brain structures. We counted co-expression sites of orexin and c-Fos manually and calculated the co-expression rate in orexin neurons.

Immunohistofluorescence analysis of ERK phosphorylation was performed following the same protocol. We used a phospho-p44/42 MAPK (Erk1/2) antibody (1:1,000 dilution; CST) as a primary antibody and an Alexa Fluor-594-labeled donkey anti-rabbit IgG (Molecular Probes) as a secondary antibody. The sections were finally examined under a microscope (BZ-X700, Keyence, Osaka, Japan).

To detect STAT3 phosphorylation, brain sections were quenched for 10 min with 3% H_2O_2 in methanol, then exposed to 3% normal donkey serum for 2 h at room temperature and incubated overnight at 4 °C with a rabbit antibody to phospho-STAT3 (1:1,000 dilution; CST). After the sections were washed with PBS, immune complexes were detected using a VECTASTAIN ABC HRP kit (rabbit IgG; Vector Laboratories, Burlingame, CA, USA). The sections were stained with a peroxidase stain DAB kit (Nacalai Tesque, Kyoto, Japan) and metal enhancer for DAB stain (Nacalai Tesque) and examined under a microscope.

2.7. Measurement of leptin concentrations

Leptin concentrations were measured in mouse MBH and plasma samples using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommended protocols.

2.8. Statistical analysis

Results were expressed as the means ± standard error of the mean (SE). Data from two groups were analyzed using the Student's *t*-test. Data from more than two groups were analyzed using one-way analysis of variance, followed by the Bonferroni test. P-values of <0.05 were considered statistically significant.

3. Results

3.1. Exercise enhances leptin sensitivity via activation of orexin neurons

We first examined whether transient treadmill running activates orexin neurons in mice. We made the mice run on a treadmill for 45 min, followed by 45 min of rest, and then took brain samples for immunohistochemical staining of orexin and c-Fos, which is a marker for excitation in neurons [20]. Compared with the sedentary control, treadmill running significantly increased the ratio of c-Fos-expressing orexin neurons to total orexin neurons (Fig. 1A–C). This result demonstrated that transient aerobic exercise activated orexin neurons in mice.

To examine leptin sensitivity in MBH after exercise, we injected mice ip with a submaximal dose of leptin after treadmill running and measured STAT3 phosphorylation in MBH of the mice using western blotting (Fig. 1D). As shown in Fig. 1E, STAT3 phosphorylation in MBH was stimulated by ip injection of leptin but not by exercise. The ip injection of leptin after exercise significantly enhanced the phosphorylation of STAT3 in MBH, suggesting that exercise may enhance leptin sensitivity in MBH.

Immunohistochemical staining showed that leptin-induced STAT3 phosphorylation was enhanced after exercise not only in the arcuate hypothalamus but also in VMH (Fig. 1F–I). Since the orexin type 1 receptor is abundantly expressed in VMH [18], we injected SB334867, an orexin type 1 receptor antagonist, into the lateral ventricle of mice before treadmill running and measured the STAT3 phosphorylation in MBH of the mice. The enhancement of STAT3 phosphorylation by exercise and leptin was significantly attenuated by icv injection of SB334867 before exercise. These results suggest that exercise-induced orexin enhances leptin sensitivity in MBH of mice via the orexin type 1 receptor.

3.2. Orexin indirectly enhances STAT3 phosphorylation induced by injection of leptin after exercise

We next determined whether orexin itself enhances leptin sensitivity in MBH, even in the sedentary state. In this experiment, we injected orexin into the lateral ventricle, and 45 min later (same as the treadmill running time), we injected leptin ip to the mice (Fig. 2A). The icv injection of orexin did not stimulate STAT3 phosphorylation, but co-administration of leptin after orexin icv injection significantly enhanced STAT3 phosphorylation in MBH (Fig. 2B). We next evaluated whether orexin directly enhances STAT3 phosphorylation in VMH where the orexin type 1 receptor is abundantly expressed. Compared with contralateral injection of saline to VMH, local unilateral injection of orexin to VMH suppressed the leptin-induced STAT3 phosphorylation (Fig. 2C). These results indicated that orexin indirectly enhances leptin sensitivity in MBH.

3.3. Exercise does not enhance leptin receptor signaling

To examine whether the exercise-induced enhancement of leptin sensitivity in MBH depends on leptin receptor signaling, we injected leptin into the lateral ventricle of mice after exercise (Fig. 3A). The icv injection of leptin, with or without treadmill exercise, did not show a significant difference in STAT3 phosphorylation (Fig. 3B), suggesting that exercise does not enhance leptin receptor signaling in MBH.

3.4. Orexin type 1 receptor is involved in exercise-induced leptin transport into MBH associated with ERK phosphorylation

A recent study has demonstrated that ERK phosphorylation in tanycytes of MBH, including the median eminence (ME), is involved in leptin transport through BBB [21]. We thus tried to detect a difference in ERK phosphorylation in MBH of mice (Fig. 4A). Immunohistochemical staining showed that the increase in ERK phosphorylation by exercise was especially obvious in the arcuate hypothalamus and ME (Fig. 4B–E). Compared with control (Fig. 4B), ERK phosphorylation was observed in the arcuate hypothalamus and ME of the exercised leptin-injected mice but not in that of the non-exercised leptin-injected mice (Fig. 4C and D). Moreover, icv injection of SB334867 before treadmill running attenuated ERK phosphorylation in the arcuate hypothalamus and ME of the mice (Fig. 4E). Additionally, we confirmed that there was no effect on the BBB permeability, which was measured by ip administration of Evans blue (data not shown).

We finally examined whether leptin was transported into MBH by exercise-induced activation of the orexin type 1 receptor. We measured leptin concentrations in MBH and plasma after exercise. The concentrations of leptin in MBH and plasma were significantly increased by ip injection of leptin, although treadmill running had no synergistic effect on leptin concentrations in MBH. In contrast, icv injection of SB334867 diminished the increase in MBH leptin after exercise, and significantly enhanced the increase of leptin in plasma (Fig. 4F and G). On the other hand, the ratio between the MBH and plasma leptin levels in exercised mice was significantly higher than in non-exercised mice. Further, SB334867 injection lowered the ratio of leptin levels in the MBH and plasma (Fig. 4H). These results suggest that the orexin type 1 receptor is involved in exercise-induced leptin transport into MBH associated with ERK phosphorylation.

4. Discussion

Orexin-producing neurons abundantly exist in the lateral hypothalamic field and are projected to almost all areas of the brain, including VMH [12,22]. Previously, we have reported that orexin injection into VMH stimulated the muscle glucose uptake via sympathetic nerve activation. We thus thought that the orexin-induced enhancement of leptin sensitivity in MBH after exercise was a direct effect of orexin in MBH. However, direct injection of orexin into MBH inhibited the enhancement of STAT3 phosphorylation by leptin after exercise. Orexin type 1 receptor signaling activates the mitogen-activated protein kinase pathway [23], which negatively regulates leptin signaling. These findings suggest the existence of a molecular mechanism through which orexin indirectly enhances leptin sensitivity in MBH.

The cause of leptin resistance is the attenuated phosphorylation of STAT3 in MBH, which is due to the impaired transit of peripheral leptin across BBB and/or to impaired leptin signaling following receptor binding [24,25]. To examine whether exercise enhances leptin receptor signaling or BBB permeability for leptin, we injected leptin in mice icv, with or without exercise, and observed similar

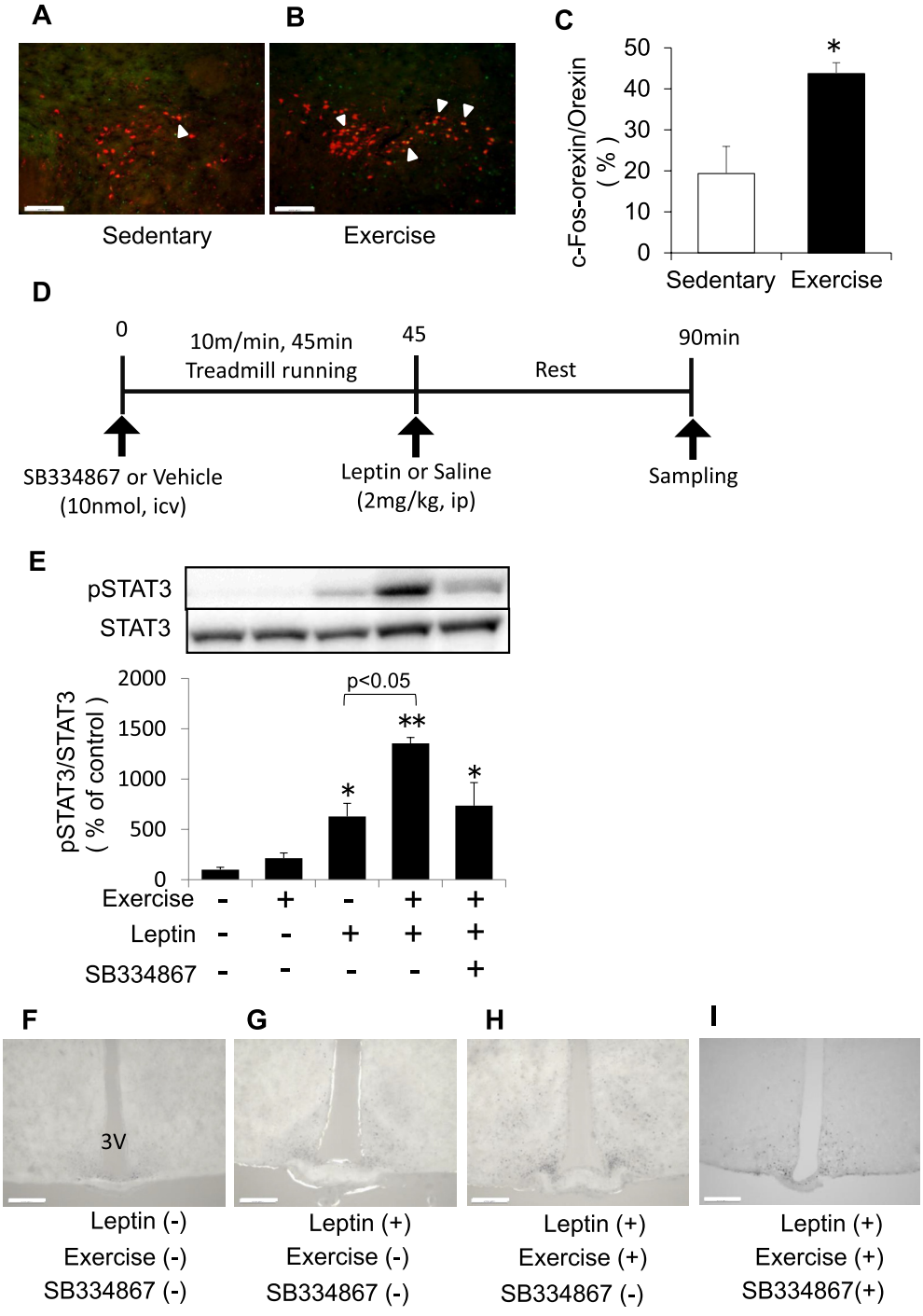

Fig. 1. Exercise enhances leptin sensitivity via activation of orexin neurons. Co-expression of orexin (red) and c-Fos (green) in the (**A**) sedentary group (n = 3) and (**B**) exercise group (n = 3). Arrow head indicates c-Fos expressing orexin neuron. (**C**) Ratio of c-Fos-expressing orexin neurons to no c-Fos-expressing orexin neurons. The values shown are the means ± SE. (**D**) Protocol used in this experiment. (**E**) Representative immunoblot phosphorylated or total STAT3 are shown above. Quantitative data are shown at bottom as bar graph (n = 4). Representative immunohistochemical staining in phosphorylated STAT3 in (**F**) control, (**G**) leptin, (**H**) exercise and leptin, and (**I**) exercise, leptin, and SB334867 groups. Scale bars = 200 μm. The values shown are the means ± SE. *P < 0.05 and **P < 0.01 vs. control. 3V: 3rd ventricle.

increases in STAT3 phosphorylation in MBH of exercised and non-exercised mice. Although we did not evaluate the activity of negative regulators of leptin receptor signaling, such as suppressor of cytokine signaling 3 and protein-tyrosine phosphatase 1B [26–28], these results suggest that the leptin receptor signaling for STAT3 might not contribute to the enhancement of leptin sensitivity in MBH, associated with exercise.

Since circulating leptin cannot easily pass through BBB, a blood-to-brain transportation system is needed for leptin. As phosphorylation of ERK in tanycytes, including ME, promotes hypothalamic STAT3 phosphorylation by increasing the transportation of circulating leptin into the brain [21], and orexin has been reported to promote ERK phosphorylation [23], we speculated that activation of orexin neurons by exercise might be involved in the enhancement of ERK phosphorylation. In this study, icv injection of SB334867 before exercise did not enhance the exercise-induced

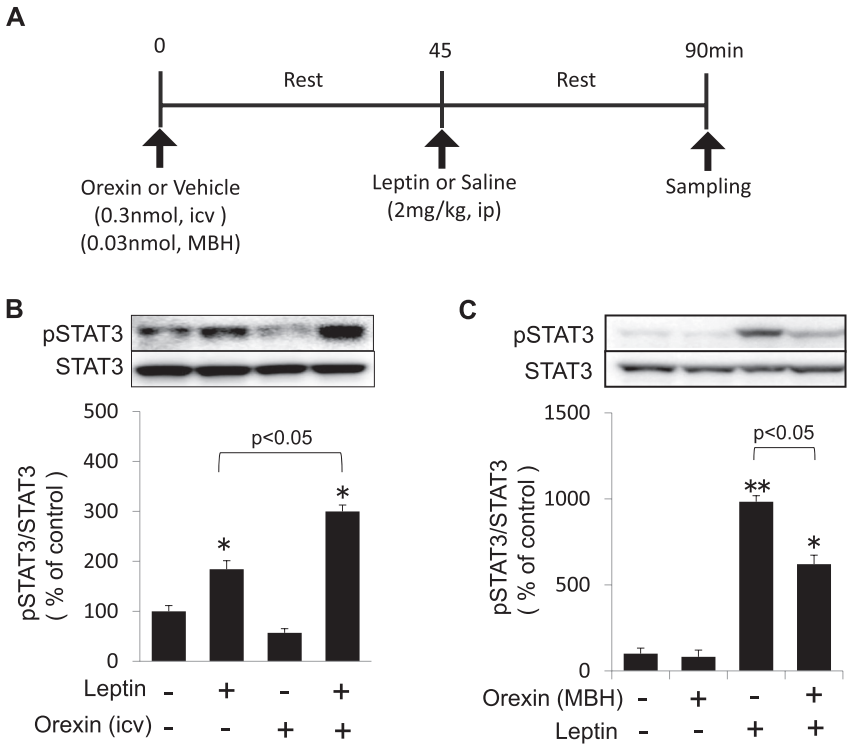

Fig. 2. Orexin indirectly enhances STAT3 phosphorylation induced by leptin after exercise. (**A**) Protocol followed in the experiments. Effect of icv (**B**) or direct injection into MBH (**C**) of orexin on STAT3 phosphorylation. Representative immunoblot of phosphorylated or total STAT3 are shown above. Quantitative data are shown at bottom as bar graph (n = 4). The values shown are the means ± SE. *P < 0.05 and **P < 0.01 vs. control.

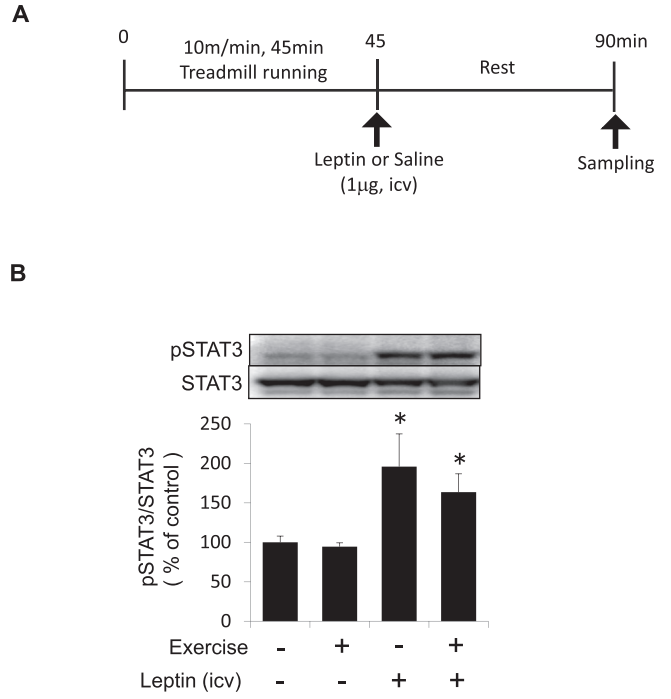

Fig. 3. Central leptin did not affect STAT3 phosphorylation in MBH. (**A**) Protocol followed in the experiments. (**B**) Representative immunoblot with phosphorylated or total STAT3 is shown above. Quantitative data are shown at bottom as bar graph (n = 4). *P < 0.05 vs. control.

ERK phosphorylation in MBH and ME of the mice. We also confirmed that treadmill exercise did not alter the BBB permeability. These results suggest that exercise-activated orexin stimulates ERK phosphorylation in MBH.

We supposed that the exercise-induced phosphorylation of ERK by orexin accelerates leptin transport into MBH. While leptin concentrations were increased in MBH by ip administration of leptin, icv injection of an orexin type 1 receptor antagonist significantly decreased the leptin concentration in MBH. However, leptin levels in MBH were not enhanced by exercise after leptin administration, even though STAT3 phosphorylation was enhanced. On the contrary, leptin levels tended to decrease in the plasma of exercised mice compared with those of non-exercised mice, although plasma leptin levels increased after ip administration of leptin. As such, the ratio between leptin level in MBH and plasma in leptin-injected groups were similar to the relative results with STAT3 phosphorylation in MBH in the same groups. Leptin bound to the long-form leptin receptor, OB-Rb, is internalized by endocytosis and degraded in lysosomes [29,30]. Generally, exercise stimulates the blood flow and decreases the plasma leptin level by inhibiting leptin secretion from adipocytes [31,32]. Moreover, adrenaline secreted during exercise stimulates leptin transport via α1-adrenergic receptors [33]. We, therefore, think that the metabolic turnover of leptin may increase in the whole body, leading to a decrease in the excess leptin level in MBH of leptin-injected exercised mice. A significant increase of the leptin level in the plasma upon SB334867 administration supported this assumption because leptin that was not transported into MBH could be accumulated in circulation.

Exercise results in much energy consumption and exerts various physiological effects on peripheral organs and the central nervous system. Here, we found that exercise contributes to the enhancement of leptin sensitivity in MBH. These data are important for understanding the effect of exercise on the improvement of leptin resistance in obese or diabetic patients.

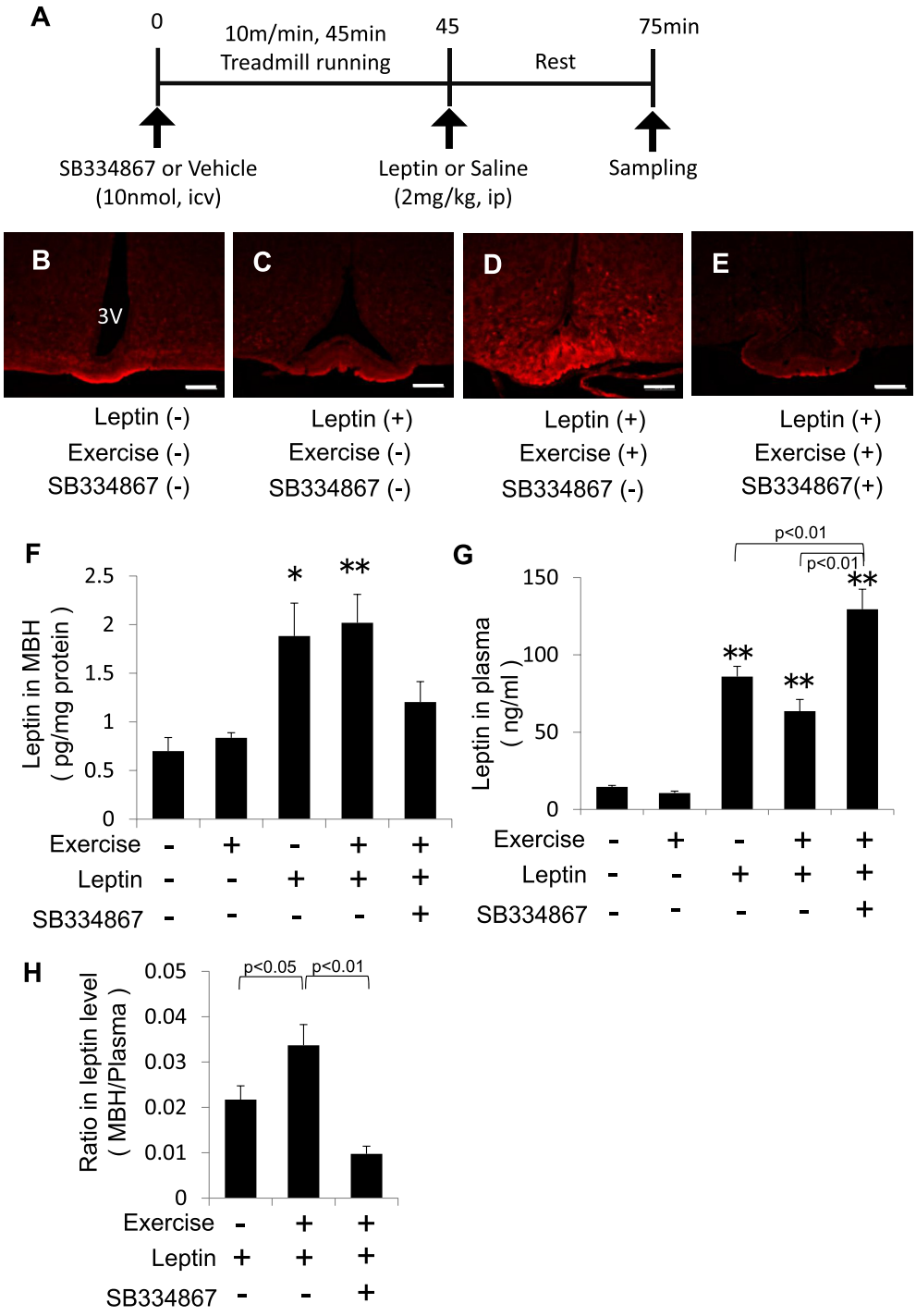

Fig. 4. Orexin type 1 receptor is involved in exercise-induced leptin transport into MBH associated with ERK phosphorylation. (**A**) Protocol followed in the experiments. Representative immunohistochemical staining in phosphorylated ERK expression in (**B**) control, (**C**) leptin, (**D**) exercise and leptin, and (**E**) exercise, leptin, and SB334867 groups. Scale bars = 100 μm. Concentrations of leptin in MBH (**F**) and in plasma (**G**). (**H**) Ratio of leptin level in MBH and plasma (n = 6). The values shown are the means ± SE. *P < 0.05 and **P < 0.01 vs. control.

Competing financial interests

The authors declare no conflicts of interest, financial or otherwise.

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