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Exercise-induced enhancement of insulin sensitivity is associated with accumulation of M2-polarized macrophages in mouse skeletal muscle

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ABSTRACT

Exercise enhances insulin sensitivity in skeletal muscle, but the underlying mechanism remains obscure. Recent data suggest that alternatively activated M2 macrophages enhance insulin sensitivity in insulin target organs such as adipose tissue and liver. Therefore, the aim of this study was to determine the role of anti-inflammatory M2 macrophages in exercise-induced enhancement of insulin sensitivity in skeletal muscle. C57BL6J mice underwent a single bout of treadmill running (20 m/min, 90 min). Twenty-four hours later, *ex vivo* insulin-stimulated 2-deoxy glucose uptake was found to be increased in plantaris muscle. This change was associated with increased number of CD163-expressing macrophages (i.e. M2-polarized macrophages) in skeletal muscle. Systemic depletion of macrophages by pretreatment of mice with clodronate-containing liposome abrogated both CD163-positive macrophage accumulation in skeletal muscle as well as the enhancement of insulin sensitivity after exercise, without affecting insulin-induced phosphorylation of Akt and AS160 or exercise-induced GLUT4 expression. These results suggest that accumulation of M2-polarized macrophages is involved in exercise-induced enhancement of insulin sensitivity in mouse skeletal muscle, independently of the phosphorylation of Akt and AS160 and expression of GLUT4.

1. Introduction

Exercise reduces blood glucose levels in diabetics [1,2] and prevents the development of type 2 diabetes [3]. These beneficial effects of exercise are mainly due to the improvement of insulin sensitivity in skeletal muscle [1,4,5]. A single bout of exercise is known to increase insulin sensitivity and insulin responsiveness of skeletal muscle in human and rodents [4,6], although the underlying mechanism of this phenomenon is not fully elucidated.

Recent studies have provided evidence for a pathophysiological link between the accumulation of macrophages in insulin sensitive tissues and insulin resistance [7–12]. Various stimuli activate macrophages to express distinct profiles of chemokines, surface markers and metabolic enzymes, which in turn induce diverse functions. Based on their distinct functions under inflammatory and anti-inflammatory conditions, these macrophages are collectively categorized into classically activated M1 and alternatively activated M2 macrophages, respectively [13,14]. In both human and rodents, macrophages accumulate in adipose tissue with increasing body weight, and their accumulation correlates with insulin resistance [7–11]. On the other hand, weight loss leads to a reduction in both inflammatory markers and macrophage content in adipose tissue [11]. In the above cases, accumulation of M1 macrophages in adipose tissue seems to have a causal role in the induction of insulin resistance [7,9,10]. Indeed, adipose tissue macrophages are the main source of proinflammatory cytokines such as TNF-α and interleukin (IL)-6 that can block insulin action in adipocytes, providing potential link between macrophages and insulin resistance [7].

On the other hand, several reports suggested that the alternatively activated M2 macrophages are required to maintain normal insulin sensitivity [7,15,16]. M2 macrophages act as "anti-inflammatory macrophages", generating high levels of anti-inflammatory molecules, such as IL-10 and IL-1 decoy receptor and expressing CD206 and CD163 on their cell surface [14].

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Peroxisome proliferator-activated receptor γ (PPARγ is a major PPAR subtype expressed in murine macrophages, and macrophage-specific PPARγ deletion impairs alternative activation of macrophages and results in decreased insulin sensitivity in both skeletal muscle and liver [15,16]. These results suggest that M2 macrophages are a positive regulator of insulin sensitivity.

Based on the above information, we hypothesized that exercise-induced enhancement of insulin sensitivity in skeletal muscle is mediated at least in part through accumulation of macrophages in skeletal muscle. To test this hypothesis, we investigated macrophage accumulation in skeletal muscle after a single bout of exercise. The results showed that a single bout of exercise induced accumulation of CD163-expressing macrophages (i.e. M2-polarized macrophages), and enhanced insulin sensitivity in the plantaris muscle. Depletion of macrophages by pretreatment with clodronate liposome (CL) abrogated these changes. These results suggest that accumulation of M2 macrophages in skeletal muscle might play a role in post-exercise enhancement of insulin sensitivity.

2. Materials and methods

2.1. Animals

Male C57BL6J mice were housed in stainless steel wire cages in a temperature-controlled clean room under a 12 h light–dark cycle. The animals were provided with standard chow and autoclaved tap water *ad libitum*. All animal experiments in this study were approved by the Animal Experimental Committee of Juntendo University.

2.2. Exercise protocol

Eight-week-old mice were randomly divided into two groups; the PBS-containing liposome (PBS)-injected group ($n = 42$) and the clodronate-containing liposome (CL)-injected group ($n = 46$). CL was obtained from Roche Diagnostics (Mannheim, Germany). Each mouse was administered 400 μl PBS or CL by intraperitoneal injection, and then randomly subdivided into the sedentary group (PBS-Sed and CL-Sed group, $n = 21$ and 23, respectively) and exercise groups (PBS-Ex and CL-Ex group, $n = 21$ and 23, respectively). Twenty-four hours later, mice in the Ex group were forced to run on a treadmill set at 10° inclination at 20 m/min for 90 min. Twenty-four hours after treadmill running, each mouse was anesthetized by a single intraperitoneal injection of nembutal (0.1 mg/g body weight) after fasting for 6 h. Then, the plantaris muscle was dissected out quickly and used for the measurement of insulin stimulated 2-deoxyglucose uptake (PBS-Sed, PBS-Ex, CL-Sed and CL-Ex groups, $n = 8$, 8, 10 and 10, respectively). The soleus, plantaris and gastrocnemius muscles were dissected out from 16 mice, and then frozen in isopentane cooled with liquid nitrogen for immunohistochemistry. The plantaris muscle was also dissected out from the remaining mice and snap-frozen in liquid nitrogen after adequate treatment as described below for Western blotting (5 mice per group) and real-time PCR (4 mice per group).

2.3. Ex vivo muscle incubation and 2-deoxyglucose uptake

The harvested plantaris muscles were pre-incubated for 30 min at 37 °C in 3 ml Krebs–Ringer-Bicarbonate buffer (KRB) containing 8 mM D-glucose. At the end of the pre-incubation period, the muscles were transferred to fresh KRB containing 8 mM D-glucose with or without 450 μU/ml insulin and incubated for 20 min at 37 °C. The muscles were subsequently rinsed in 3 ml KRB containing 8 mM D-mannitol at 30 °C for 10 min, and then glucose transport was measured in 2 ml KRB containing 1 mM 2-deoxy-D-[1,2-^{3}H]-glucose (1.5 μCi/ml) (2-DG) and 7 mM D-[^{14}C]-mannitol (0.3 μCi/ml) at 30 °C for 10 min. All buffers were continuously bubbled with 95% O_2–5% CO_2 gas mixture. To terminate the transport, muscles were dipped in ice-cold KRB, then blotted on a filter paper, trimmed and processed by incubation in 420 μl of 1 N NaOH at 100 °C for 5 min, neutralized with 70 μl of 6 N HCl, and particulates were precipitated by centrifugation. Radioactivity in aliquots of digested muscle protein was determined by liquid scintillation counting for dual labels, and 2-DG uptake was calculated as described previously [17]. For Western blotting, the muscle was incubated *ex vivo* in the same manner as mentioned above except for the lack of the radioisotopes.

2.4. Western blotting

For phospho-Akt, total-Akt, phospho-AS160, total AS160 and CD163 protein analysis, dissected plantaris muscles were homogenized and analyzed as described previously [18].

2.5. Total membrane fractionation and measurement of GLUT4 content

The plantaris muscles obtained from mice of the PBS-Sed, PBS-Ex, CL-Sed and CL-Ex groups were homogenized separately in 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 400 mM PMSF and Halt protease inhibitor cocktail (Thermo Fisher Scientific, MA) in Protein LoBind tubes (Eppendorf, Hamburg, Germany). Homogenates were centrifuged at 1000×*g* for 10 min, and the supernatants were collected. The resulting pellet was resuspended in the buffer, rehomogenized as described above, and recentrifuged at 1000×*g* for 10 min. The resulting pellet was discarded, and the supernatant was combined with the first supernatant and centrifuged at 9000×*g* for 10 min. The resulting supernatant was centrifuged at 190,000×*g* for 60 min, and the resulting pellet was resuspended in 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) for measurement of protein concentration, and heat-denatured in sample buffer. GLUT4 content was visualized by Western blotting.

2.6. Immunohistochemistry

Transverse sections (8-μm thick) of the plantaris muscle of 16 mice were prepared with a cryostat cooled to below −16 °C. The sections were dried in air for 30 min, and then fixed with 4% paraformaldehyde for 10 min at room temperature. After three washes with PBS, the sections were incubated in 0.1% Triton-X100 in PBS for 15 min. M2-polarized macrophages were identified by overnight incubation at 4 °C with a rat anti-mouse F4/80 antibody (Biolegend Japan, Tokyo, Japan) and rabbit anti-mouse CD163 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Then, the sections were washed with PBS-T and incubated with an Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 594-conjugated anti-rat IgG antibody (Life Technology Japan, Tokyo, Japan) for 30 min at room temperature. Sections were washed with PBS-T and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and glass coverslips. Images were captured with a FV1000 confocal microscope and FV10-ASW ver 1.6 software (Olympus, Tokyo). The total surface area of each muscle section was analyzed and labeled cells were expressed as the total area they occupied (mm^2).

2.7. RNA isolation, reverse transcription and real-time PCR

Total RNA isolation, first-strand cDNA generation and real-time PCR were performed as previously described [19]. Signals were normalized against cyclophilin mRNA.

2.8. Statistical analysis

All data were expressed as mean ± SD. Differences between groups were analyzed by one-way analysis of variance (ANOVA). A p value of less than 0.05 was considered to denote a statistically significant difference.

3. Results

3.1. A single exercise session enhances 2-deoxyglucose uptake and accumulation of F4/80$^+$CD163$^+$ macrophages in plantaris muscle

Several studies have reported that a single session of exercise enhances muscle insulin sensitivity [4,6]. We also observed enhanced insulin-stimulated 2-deoxyglucose (DG) uptake in plantaris muscle 24 h after a single bout of exercise in PBS-treated mice (Fig. 1A and B). We also observed comparable lactate contents in plantaris muscle before and after *ex vivo* incubation (data not shown). In addition, basal and insulin stimulated 2-DG uptake levels were similar to a previous study using mouse extensor digitorum longus (EDL) muscle [20]. These data suggest that the viability of skeletal muscle was not impaired during our procedure. To determine whether this enhancement of 2-DG uptake is associated with accumulation of macrophages in skeletal muscle, we examined the presence of macrophages by immunohistochemistry of frozen sections using an anti-F4/80 antibody (a pan marker of macrophages) and anti-CD163 antibody (a marker of M2 macrophages). As shown in Fig. 2A and B (PBS-Sed), a few F4/80-positive cells were found in the plantaris muscle at rest and most of these cells also expressed CD163 (F4/80$^+$CD163$^+$). The numbers of F4/80$^+$CD163$^+$ cells increased at 24 h after a single exercise session (Fig. 2A and B, PBS-Ex), while F4/80$^+$CD11c$^+$ cells (M1 macrophages) were barely detectable upon immunohistochemistry both at rest and after exercise (data not shown). Consistent with these findings, CD163 protein expression (Fig. 2C) as well as mRNA expression of M2 marker genes such as CD206, arginase-1 and dectin-1, were increased 24 h after exercise (Fig. 2D). These findings suggest that increased insulin sensitivity after a single exercise session is associated with accumulation of CD163-positive macrophages in the skeletal muscle.

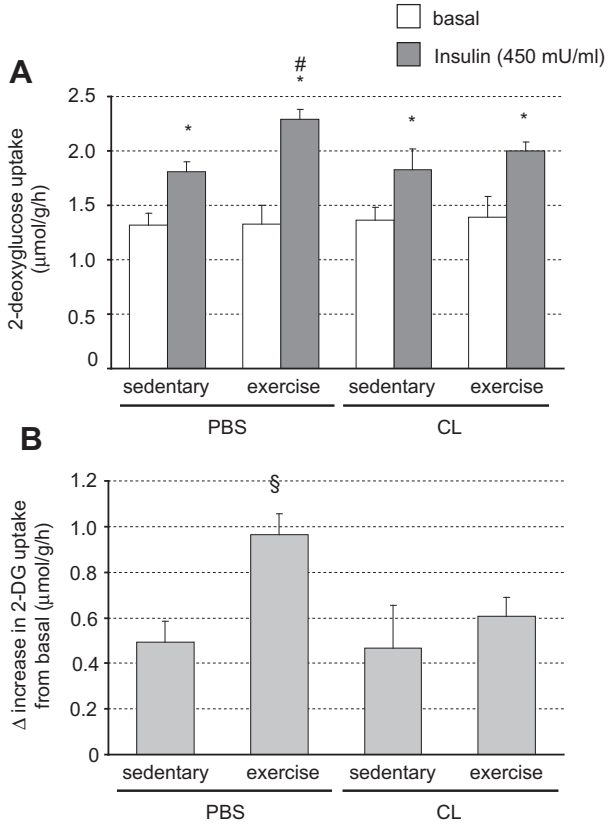

Fig. 1. Injection of CL abrogates the exercise-induced increase in insulin responsiveness in mouse plantaris muscle. (A) After injection of PBS or CL, mice were divided into the sedentary and exercise groups. Twenty-four hours after a single session of exercise or sedentariness, we measured ex vivo insulin-stimulated 2-DG uptake in plantaris muscle at basal (white bars) and insulin (450 μU/ml)-stimulated (gray bars) states. (B) Delta increase in 2-DG uptake. Data are shown as mean ± SD of 8 (PBS group) or 10 (CL group) mice. $^*p < 0.01$ vs. basal value. $^{**}p < 0.05$ vs. insulin-stimulated state in the sedentary group. $^{***}p < 0.01$ vs. all other groups.

3.2. Pretreatment with CL abrogates exercise-induced enhancement of 2-DG uptake and accumulation of F4/80$^+$CD163$^+$ macrophages in plantaris muscle

To test the role of macrophages in exercise-induced enhancement of insulin sensitivity, clodronate-containing liposomes (CL), which systemically deplete macrophages, were injected 24 h before exercise [21,22]. Twenty-four hours after CL treatment, the numbers of F4/80$^+$CD163$^+$ cells were not altered in the non-exercised muscles (Fig. 2A and B, CL-Sed). However, the accumulation of F4/80$^+$CD163$^+$ cells in skeletal muscle after exercise was inhibited by CL treatment (Fig. 2A and B, CL-Ex). Consistently, CL treatment abolished the post-exercise increase in mRNA expression levels of CD206, arginase-1 and dectin-1 (Fig. 2D). The same treatment also simultaneously canceled the associated exercise-induced enhancement in insulin responsiveness (Fig. 1). These results suggest that accumulation of F4/80$^+$CD163$^+$ macrophages in skeletal muscle plays a role in enhancement of insulin sensitivity in skeletal muscle after a single exercise session.

3.3. A single exercise session and CL treatment does not alter insulin-stimulated phosphorylation of Akt and AS160

To further investigate the mechanism of exercise-induced enhancement of insulin sensitivity associated with F4/80$^+$CD163$^+$ macrophage accumulation, we evaluated the insulin-stimulated phosphorylation of Akt and AS160, which occurs downstream of insulin signal transduction, by Western blotting. As shown in Fig. 3, treatment with insulin enhanced phosphorylation of Akt (Thr308 and Ser473) and AS160 (Thr642) in the plantaris muscle, whereas neither a single exercise session nor CL treatment significantly altered this basal or insulin-stimulated phosphorylation of Akt and AS160.

3.4. A single exercise session increases plantaris muscle GLUT4 content at 24 h after a single bout of exercise in both PBS- and CL-treated mice

Upregulation of GLUT4 expression is associated, at least in part, with the insulin sensitivity of skeletal muscles [23,24]. We therefore finally compared GLUT4 expression levels in the plantaris muscle of sedentary and 24 h post-exercise mice treated with PBS and CL. Consistent with previous studies [24], a single exercise session increased GLUT4 content in the skeletal muscle (Fig. 4, PBS-Sed vs. PBS-Ex). Similarly, GLUT4 expression levels were also increased 24 h after exercise in CL-treated mice (Fig. 4, CL-Sed vs. CL-Ex).

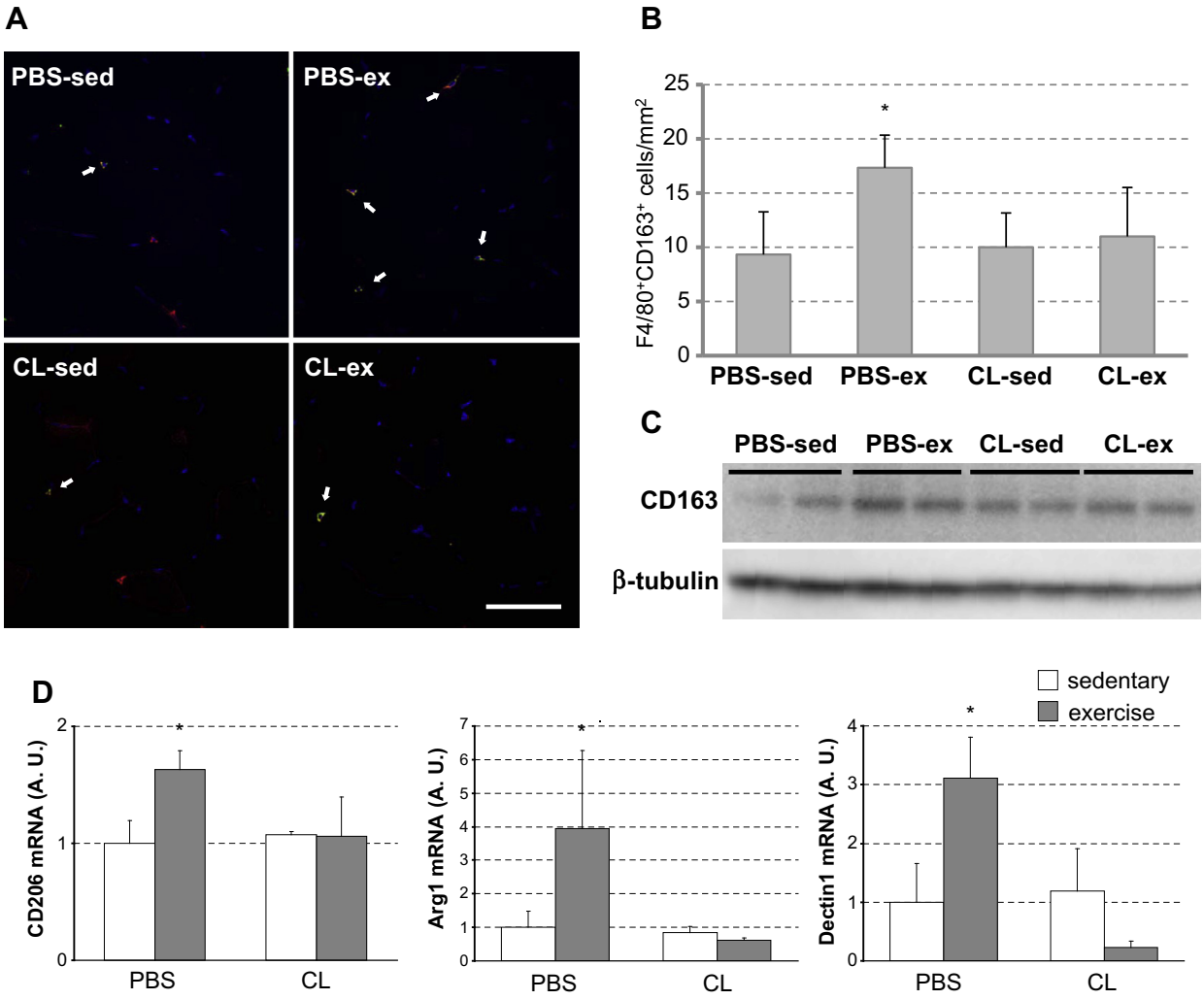

Fig. 2. Injection of CL inhibits accumulation of F4/80$^+$CD163$^+$ cells in skeletal muscle after exercise. (A) Representative immunostained images using an anti-F4/80 antibody (red) and anti-CD163 antibody (green) of mouse skeletal muscle from the PBS-treated sedentary group (PBS-Sed), PBS-treated exercise group (PBS-Ex), CL-treated sedentary group (CL-Sed) and CL-treated exercise group (CL-Ex). Superimposed images indicate M2-polarized macrophages (arrows). Scale bars = 50 μm. (B) F4/80$^+$CD163$^+$ cell populations in mouse skeletal muscle (soleus, plantaris and gastrocnemius) from PBS-Sed, PBS-Ex, CL-Sed and CL-Ex. (C) Representative Western blot analysis of plantaris lysates from the PBS-Sed, PBS-Ex, CL-Sed and CL-Ex group using anti-CD163 and β-tubulin antibodies. (D) Relative mRNA expression of CD206, arginase-1 and dectin-1 in plantaris muscle from the PBS-Sed, PBS-Ex, CL-Sed and CL-Ex groups.

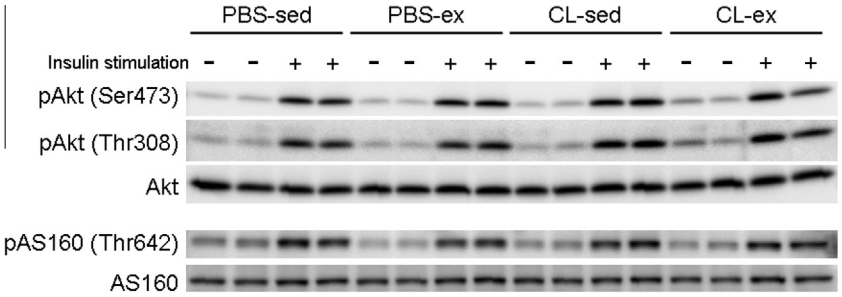

Fig. 3. Exercise and CL treatment do not affect the phosphorylation state of Akt/AS160 in plantaris muscle. Plantaris muscle lysates were Western blotted with antibodies against phospho-Ser473 and Thr308-Akt, phospho-Ser642-AS160, and total Akt and AS160 (as loading controls). Experiments were repeated twice, and results of a representative experiment are shown.

4. Discussion

Previous studies indicated that a single bout of exercise enhances muscle insulin sensitivity in healthy and insulin-resistant human/rodents [4,25,26], although the underlying mechanism remains unresolved. In the present study, we demonstrated that exercise-induced enhancement of insulin sensitivity in mouse skeletal muscle is associated with accumulation of CD163-positive M2-polarized macrophages. The results also showed that depletion of macrophages by CL abolishes the increase in insulin-stimulated

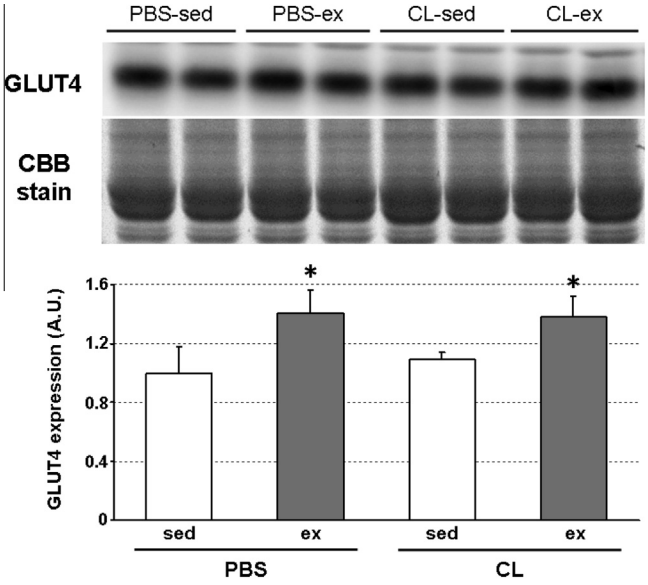

Fig. 4. GLUT4 expression increased after exercise in the plantaris muscle of both PBS- and CL-treated mice. GLUT4 immunoblotting (top panel) and Coomassie Brilliant Blue staining of the running gel as a loading control (bottom panel). The PBS-Sed group was assigned a value of 1.0; all other values are expressed relative to this value. Data represent mean ± SD of 5 mice per group. $^{*}p < 0.05$, vs. PBS-Sed group.

2-DG uptake at 24 h after exercise. These results suggest that accumulation of CD163-positive M2-polarized macrophages in muscles plays a role in exercise-induced enhancement of insulin sensitivity. However, this action seems to be independent of the phosphorylation of Akt and AS160, and expression of GLUT4.

Macrophages accumulate in adipose tissue [8,9,16,27] and skeletal muscle [16,27] upon increases in body weight. Under such conditions, the majority of increased macrophages are F4/80- and CD11c-positive, proinflammatory M1 macrophages [7,9]. On the other hand, our results show an increased number of F4/80^{+}CD163^{+} cells (CD163 is a marker of M2 macrophages) in skeletal muscle at 24 h after exercise, while F4/80^{+}CD11c^{+} cells were barely detectable upon immunohistochemistry both at rest and after exercise. Similarly, Przybyla et al. showed the dominant presence of F4/80^{+}CD163^{+} macrophages in skeletal muscle at rest and that the number increased at 72 h after resistance exercise in humans [28]. Considered together, the abundantly present macrophages in post-exercise skeletal muscle are the M2 phenotype both in human and mice.

Experimental evidence suggests that M2 macrophages can regulate insulin sensitivity in insulin target organs [7]. PPARγ regulates M2 polarization in macrophages, and deletion of the PPARγ gene in macrophages results in a marked decrease of M2 macrophages in whole body and induces skeletal muscle and hepatic insulin resistance in lean mice [15,16]. Furthermore, Odegaard et al. suggested that alternative activation of Kupffer cells, the resident macrophages in the liver, prevents obesity-induced insulin resistance and lipid metabolism abnormities in the liver [29]. Based on these reports, M2 macrophages are considered to be positive regulators of insulin sensitivity and to play a role in the maintenance of normal insulin sensitivity in skeletal muscle and liver. In the present study, systemic depletion of macrophages by the administration of CL abrogated the exercise-induced accumulation of M2-polarized macrophages and cancelled the enhancement of insulin sensitivity in skeletal muscle. These results indicate the importance of M2-polarized macrophage accumulation in the muscle for the maintenance of normal insulin sensitivity as well as enhancement of insulin sensitivity in skeletal muscle after a single bout of exercise. Since injection of CL depleted macrophages throughout the body, we cannot exclude the possible role of macrophages located in tissues other than muscle, in muscle insulin sensitivity.

Overexpression of GLUT4 in skeletal muscle is reported to enhance insulin-stimulated glucose uptake [23], and one session of exercise increase GLUT4 protein expression in skeletal muscle at 16 h after exercise [24]. Thus, upregulation of GLUT4 in skeletal muscle appears to be involved in enhanced insulin sensitivity after brief exercise. In agreement with the above report, our study showed that a single session of exercise increases GLUT4 content in the plantaris muscle, and that this increase is still observed in CL-treated mice, although CL abrogates enhanced insulin sensitivity at 24 h after exercise. Furthermore, consistent with previous findings [30–32], our results demonstrated no changes in insulin-stimulated phosphorylation of Akt and AS160 after a single bout of exercise. Thus, it remains unclear as to how macrophages contribute to the enhanced insulin sensitivity observed after brief exercise. It has been suggested that factors secreted by resident M2 macrophages may directly modulate oxidative metabolism in the liver [29]. Therefore, it is possible that certain cytokines (e.g. IL-10) derived from M2 macrophages may, at least in part, contribute to the enhancement of insulin sensitivity in skeletal muscle [33]. In addition, increased insulin sensitivity can be explained, at least in part, by increased insulin-stimulated sarcolemmal GLUT4 content [34]. It is also possible that the exercise-induced increase in insulin sensitivity is mediated through macrophage-induced activation of GLUT4 trafficking to the plasma membrane. Further studies are required to understand the role of M2 macrophage accumulation in post-exercise insulin sensitivity.

In conclusion, our results suggest that accumulation of M2-polarized macrophages is involved in the exercise-induced enhancement of insulin sensitivity in mouse skeletal muscle.

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