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# Macrophage migration inhibitory factor diminishes muscle glucose transport induced by insulin and AICAR in a muscle type-dependent manner

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ABSTRACT

Skeletal muscle is a primary organ that uses blood glucose. Insulin- and 5′AMP-activated protein kinase (AMPK)-regulated intracellular signaling pathways are known as major mechanisms that regulate muscle glucose transport. It has been reported that macrophage migration inhibitory factor (MIF) is secreted from adipose tissue and heart, and affects these two pathways. In this study, we examined whether MIF is a myokine that is secreted from skeletal muscles and affects muscle glucose transport induced by these two pathways. We found that MIF is expressed in several different types of skeletal muscle. Its secretion was also confirmed in C2C12 myotubes, a skeletal muscle cell line. Next, the extensor digitorum longus (EDL) and soleus muscles were isolated from mice and treated with recombinant MIF in an *in vitro* muscle incubation system. MIF itself did not have any effect on glucose transport in both types of muscles. However, glucose transport induced by a submaximal dose of insulin was diminished by co-incubation with MIF in the soleus muscle. MIF also diminished glucose transport induced by a maximal dose of 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), an AMPK activator, in the EDL muscle. These results suggest that MIF is a negative regulator of insulin- and AICAR-induced glucose transport in skeletal muscle. Since MIF secretion from C2C12 myotubes to the culture medium decreased during contraction evoked by electrical stimulations, MIF may be involved in the mechanisms underlying exercise-induced sensitization of glucose transport in skeletal muscle.



## 1. Introduction

Skeletal muscle has recently been recognized as a secretory organ. Pedersen and Febbraio suggested that cytokines and other peptides that are produced, expressed, and released by muscle fibers and exert autocrine, paracrine, or endocrine effects should be classified as myokines [1]. For example, some interleukins (IL-4, IL-6, IL-7, IL-8, and IL-15), growth factors (insulin-like growth factor-1, fibroblast growth factor (FGF)-2, and FGF-21), and other molecules (myostatin, brain-derived neurotrophic factor, follistatin-related protein 1, irisin, and leukemia inhibitory factor) [1] have been identified as myokines. Among these molecules, IL-6 is one of the most well-investigated myokines [2]. It has been reported that MIF secretion is regulated by muscle contraction in humans *in vivo* and in cultured muscle cells [3,4]. Acute treatment of rat L6 muscle cells with IL-6 has been shown to increase basal glucose uptake and translocation of the glucose transporter GLUT4 [5]. Moreover, IL-6 has been shown to increase intramyocellular [5] and whole-body [6] fatty acid oxidation via AMPK [5,7]. These observations suggest that myokines may be responsible for metabolic homeostasis in skeletal muscle.

MIF has emerged as an important regulator of inflammation [8,9], playing a central role in the control of both innate and antigen-specific immunity [10,11] through its receptors CD74, CXCR2, and CXCR4 [12]. Although MIF was initially described as T cell-derived, it has been recently shown to be released by a variety of cell types. Autocrine/paracrine effects of MIF have been reported in heart and adipose tissue. Miller et al. showed that MIF is released from the ischemic heart, where it stimulates AMPK activation

*Abbreviations:* MIF, macrophage migration inhibitory factor; EDL, extensor digitorum longus; AICAR, 5-aminoimidazole-4-carboxyamide ribonucleoside; AMPK, 5′AMP-activated protein kinase; IL, interleukin; TNF-α, tumor necrosis factor-α; LDH, lactate dehydrogenase; TA, tibialis anterior.

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through CD74, promotes glucose uptake, and protects the heart during ischemia-reperfusion injury [13]. Atsumi et al. showed that the insulin resistance evoked by TNF-α in adipose tissue can be largely explained by the autocrine/paracrine action of MIF [14].

In this study, we examined whether the myokine MIF affects muscle glucose transport induced by insulin or AMPK activation.

## 2. Materials and methods

### 2.1. Animals

ICR mice (8–9 weeks old) were obtained from Sankyo Lab (Tokyo, Japan). The mice were housed at 23–25 °C with a 12 h light/dark cycle. The mice received an MF certified diet (Oriental Yeast, Tokyo, Japan) and water ad libitum. Care and use of the laboratory animals were in accordance with the guidelines of the Experimental Animal Committee of Tokyo Metropolitan University and followed the Guidelines for the Proper Conduct of Animal Experiments established by the Science Council of Japan.

### 2.2. Cell culture

A mouse skeletal muscle cell line, C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA), was grown on 4-well plates (Nalge Nunc, New York, USA) at a density of $2 \times 10^5$ cells/well with 3 ml of growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM; 25 mM glucose; Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (Bio West, Nuaillé, France) and 1% penicillin–streptomycin, at 37 °C under a 5% $CO_2$ atmosphere until confluence. Differentiation was then induced by switching the growth medium to DMEM supplemented with 2% calf serum (Bio West) and 1% penicillin–streptomycin (day 0). Four days after the initiation of differentiation, the cells were used for experiments. The differentiation medium was changed every 24 h.

### 2.3. C2C12 myotube contraction by electrical stimulation

A day before the experiment, the cells were washed with PBS twice, followed by addition of 2 ml of serum and phenol red-free DMEM. After incubation for 1 h at 37 °C, the medium was changed to fresh medium again and incubated for 24 h at 37 °C. On the day of the experiment, the medium was changed again to fresh medium and the 4-well plates were placed in the electrical stimulation apparatus, a 4-well C-Dish (Ion Optix Corp., MA, USA) that was connected to an electrical stimulator (Uchida Denshi, Tokyo, Japan) and stimulated with electric pulses of 20 mA at 1 Hz for 30 ms at 970-ms intervals for 1 h in an incubator at 37 °C, as described previously [15]. After the contraction experiment, the conditioned medium was collected and cells were harvested with 300 μl of ice-cold lysis buffer. The conditioned medium was centrifuged for 15 min at 2000*g*, followed by centrifugation for 35 min at 12,000*g* at 4 °C to eliminate cell fragments. The supernatant was concentrated using 3 kDa cut-off centrifugal filters (Millipore, Watford, UK) after filtration with a 0.22-μm filter (Millipore, Watford, UK). The concentrated conditioned medium was used for immunoblotting and a LDH assay after correction for concentrate volume. The harvested cells were sonicated and centrifuged at 14,000*g* for 20 min at 4 °C, and the supernatant was used for immunoblotting.

### 2.4. Expression vector construction

Mouse MIF cDNA was cloned by a RT-PCR based method. To ensure that the cDNA was tagged with HA at its C-terminus, the forward primer used for its amplification included the *Eco*RI enzyme restriction site (GAATTC) and Kozac sequence (GCCACC) before the start codon of the coding sequence for mouse MIF (5′-GAATTCGCCACCATGTTCATCGTGAACACCAATGTT-3′). The reverse primers included recognition sites for the restriction enzymes *Xho*I (CTCGAG) and *Bgl*II (AGATCT), the complement of the HA sequence, and the complement of the coding sequence for the C-terminal end of mouse MIF (5′-AGATCTCTCGAGTCAAGCGTAATCTGGAACATCG-TATGGGTAAGCGAAGGTGGAACCGTTCCAGCCC-3′). The PCR cycling condition was 25 cycles of 98 °C for 10 s, 60 °C for 5 s and 72 °C for 10 s. Amplified fragments were cloned into the pCR-Blunt vector (Life Technologies, CA, USA), digested with the restriction enzymes *Eco*RI and *Xho*I, and then subcloned into the pCAGGS vector before analysis of the sequence. All DNA preparations were performed using the EndoFree Plasmid Mega Kit (Qiagen, Hilden, Germany), and the concentration and purity of the plasmid preparations were determined using a spectrophotometer (Thermo Fisher Scientific, MA, USA).

### 2.5. DNA injection into skeletal muscle and in vivo electroporation

DNA injection and *in vivo* electroporation were performed using a modification of the method of Aihara and Miyazaki [16]. Female mice were anesthetized intraperitoneally (100 mg of pentobarbital per kg of body weight). The plasmid DNA was diluted in 0.9% saline to a final concentration of 4 μg/μl. An insulin syringe was used to inject 25 μl or 50 μl of DNA solution intramuscularly each into the TA muscle or the gastrocnemius muscle along the long axes of the muscle fibers. Immediately after injection of the DNA, an electrode and a pair of stainless steel needles were inserted into the skeletal muscle, which was then stimulated with eight square wave electric pulses (200 V/cm), each with a frequency of 1 Hz and duration of 20 ms, delivered using an electrical pulse generator. At 2 weeks after electroporation of the muscle tissues, the serum was collected and the muscles were dissected and immediately frozen in liquid nitrogen.

### 2.6. In vitro muscle incubation and glucose transport

Male mice were sacrificed, and the EDL and soleus muscles were rapidly removed and treated for *in vitro* muscle incubation as previously described [17]. Briefly, both ends of the muscle strips (tendons) were tied with sutures and mounted on an incubation apparatus. The muscles were preincubated for 2 h in 1.3 ml of Krebs–Ringer bicarbonate buffer (KRB) containing 2 mM pyruvate with or without 1 μg/ml MIF (R&D Systems, Minneapolis, USA). The muscles were then incubated in KRB with or without 50 mU/ml or 200 μU/ml insulin (Eli Lilly, Indianapolis, USA) for 30 min, or with 2 mM or 250 μM AICAR (Wako, Osaka, Japan) for 20 min in the presence or absence of MIF. The buffers were kept at 37 °C throughout the experiment and gassed continuously with 95% $O_2$ and 5% $CO_2$.

For glucose transport, the incubated muscles were transferred to KRB containing 1 mM 2-deoxy-D-glucose (1.5 μCi/ml) and 7 mM d-[14C]mannitol (0.45 μCi/ml) (PerkinElmer Life Sciences, MA, USA) at 30 °C and incubated for 10 min. MIF, insulin, and AICAR were added to each glucose transport buffer at a concentration equal to that of the incubation buffer. Glucose transport was terminated by dipping the muscle tissue in KRB at 4 °C, and the muscle tissue was frozen in liquid nitrogen. The muscle tissue was weighed and digested by incubation in 250 μl of 1 N NaOH at 80 °C for 10 min. The digests were neutralized with 250 μl of 1 N HCl, and particulate matter was precipitated by centrifugation at 13,000*g* for 5 min. The radioactivity in aliquots of the digested muscles was determined by liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated as previously described [18].