早稲田大学審査学位論文

博士（スポーツ科学）

Investigation of the Roles of Exercise-Induced Humoral Factors and Their Receptors

運動によって分泌される液性因子および その受容体の役割の検討

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Contents

Abbreviation

ALT	alanine aminotransferase
ANOVA	analysis of variance
Areg	amphiregulin
Arg1	arginase1
AST	aspartate aminotransferase
ATF4	activating transcription factor 4
BSA	bovine serum albumin
BTC	betacellulin
BUN	blood urea nitrogen
CAT	catalase
CCR	CC chemokine receptor
CHOP	C/EBP homologous protein
CK	creatine kinase
COX IV	cytochrome c oxidase subunit IV
EGF	epidermal growth factor
EGFR	EGF receptor
EVs	extracellular vesicles
FDR	false discovery rate
FFA	free fatty acid
GADD34	growth arrest and DNA damage-inducible protein 34
GLUT4	glucose transporter 4
GST	glutathione S-transferase
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HDC	histidine decarboxylase
HDL	high-density lipoprotein
HFD	high fat diet
HO-1	heme oxygenase-1
IL	interleukin
ILC2s	type 2 innate lymphoid cells
IL-1RI	IL-1 type I receptor
IL-6R	IL-6 receptor
IRS1	insulin receptor substrate 1
KO	knock out

LDH	lactate dehydrogenase
LDL	low-density lipoprotein
MCP	monocyte chemoattractant protein
M-EVs	muscle-derived extracellular vesicles
MIP	macrophage inflammatory protein
miRNA	microRNA
MPO	myeloperoxidase
ND	normal diet
NQO	quinone dehydrogenase
Nrf	NF-E2-related factor
ORP150	oxygen-regulated protein 150
PBS	phosphate-buffer saline
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PGC-1α	peroxisome proliferator-activated receptor coactivator-1α
SE	standard error
Sirt1	sirtuin 1
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substance
Tfam	mitochondrial transcription factor A
TG	triglyceride
TGF-α	transforming growth factor-α
TLR4	toll like receptor 4
TNF	tumor necrosis factor
Tregs	regulatory T cells
VEGF-A	vascular endothelial growth factor A
XBP1	X-box binding protein 1

Chapter 1. Background

1.1. Introduction

Physical exercise has various benefits for systemic health (e.g., improvement of type 2 diabetes, preventive effects on cancer, and anti-inflammatory effects against chronic inflammation) [1]. Furthermore, exercise training induces various exercise adaptations (e.g., muscle hypertrophy, muscle angiogenesis, neural adaptation, enhancing endurance capacity, and increasing bone mineral density) [2]. A single bout of exercise also has positive effects on physical functions (e.g., improving postprandial glycemia, insulin sensitivity, and cognitive function) [3–5]. As one of the mechanisms for such exercise effects, muscle-derived humoral factors called myokine contribute to parts of these exercise effects [6]. Other than muscle, various organs secrete humoral factors, which are called depending on the source of organs (e.g., hepatokine from liver and adipokine from adipose tissue) [7,8]. Recently, various exercise-induced humoral factors have been identified and revealed to contribute a part of health-promoting effects. However, the source of exercise-induced humoral factors is mostly unidentified. Because of these backgrounds, these exercise-induced humoral factors have been reorganized and called “exerkine” recently [9]. Exerkines include various types of protein (e.g., cytokines,

chemokines, and growth factors) and extracellular vesicles (EVs) and are secreted by various types of cells (e.g., muscle cells, immune cells, fibroblasts, and satellite cells) and organs [9]. Although many exerkines have already been identified, these physiological roles are not completely understood. Also, the importance of exerkine receptors is uninvestigated. In the next section, this thesis reviewed some exerkines, their receptors, and presumable physiological roles.

1.2. Exerkines, Their Receptors, and Their Presumable Physiological Roles

1.2.1. IL-6

Interleukin (IL)-6 is a major multifunctional cytokine increased by exercise in circulation [10–14], urine [10,11,13,15], and muscle [12,16]. IL-6 is secreted by muscle cells [6], macrophages [12], adipose tissue [17], and brain [18] responding to exercise. At first, exercise-induced IL-6 is supposed to act as an inflammatory mediator because strenuous exercise induces systemic inflammation, which coincides with muscle damage [19]. However, it has been revealed that IL-6 has physiological roles other than inflammatory mediators. For example, IL-6 acts as an energy utilization regulator (e.g., glucose uptake and fat oxidation) [20,21], a glycemic modulator via glucagon-like peptide-1 secretion of intestinal L cells and pancreatic alpha cells [22,23], a suppressor

of tumor growth [24], and a mediator for muscle hypertrophy [25]. IL-6 signaling is transmitted by IL-6 receptor (IL-6R) and transmembrane protein gp130 [21]. Because the injection of IL-6R antagonist (IL-6R blocker) attenuates exercise-induced reduction of visceral adipose tissue mass [26], it is suggested that IL-6R is an important receptor for exercise effects.

1.2.2. IL-1β

IL-1β is a representative inflammatory cytokine increased by exercise in circulation [11,14,27], urine [11,14,15], and muscle [28–32]. However, it has been reported that circulating IL-1β does not change following exercise [11,33]. Recently, some studies have reported that IL-1β is important for endurance capacity [30,32]. IL-1β is produced by neutrophils during exercise and stimulates muscle glucose uptake via glucose transporter 4 (GLUT4) translocation [30,32,34]. Furthermore, IL-1β is produced by brain macrophages following exercise, and brain IL-1β induces fatigue after muscle-damaging exercise [35–37]. IL-1 type I receptor (IL-1RI) is one of the receptors for IL-1β, and its deletion reduces locomotor activity [38]. These studies suggest that IL-1β-IL-1RI axis may be important for exercise performance.

1.2.3. MCP-1

Monocyte chemoattractant protein (MCP)-1 is a major chemokine recruiting monocyte/macrophages, which increases after exercise in circulation [10,11,14,19,39], urine [10,11,13,15], and muscle [12,16,29,40,41]. MCP-1 is secreted by neutrophils after exercise [29] and by C2C12 myotubes responding to muscle contraction [42,43]. MCP-1 has chemoattractant activities for monocytes/macrophages and recruits macrophages to muscle in various muscle damage models (e.g., exercise, cardiotoxin, and traumatic puncture wounds) [44]. CC chemokine receptor 2 (CCR2) is a receptor for MCP-1 and is mainly expressed on macrophages [44,45]. Therefore, CCR2 is also an important factor for recruiting macrophages after muscle damage [44]. Because recruited macrophages contribute to muscle regeneration, MCP-1-CCR2 signaling indirectly has an important role in muscle regeneration via macrophage recruitment [44,45]. MCP-1-CCR2 signaling also directly proliferates myoblasts in vitro [46]. Furthermore, single-nucleotide polymorphisms of MCP-1 and CCR2 are associated with muscle strength [47]. These studies suggest that MCP-1 and CCR2 may be important factors for exercise-induced muscle growth.

1.2.4. IL-13

IL-13 is a Th2 cytokine and plays a central role in gastrointestinal parasite expulsion, airway hyperresponsiveness, allergic inflammation, asthma, and tissue fibrosis [48]. The changes in muscle or circulating IL-13 levels after acute exercise is controversial [39,40,49–51]. However, exercise training increases muscle and circulating IL-13 levels [39,51,52]. Recently, IL-13 has been identified as an important cytokine for exercise adaptation, such as mitochondrial biogenesis, increasing endurance capacity, and enhancing energy substrate utilization via its receptor IL-13 receptor α1 [51]. Exercise training increases the number of muscle type 2 innate lymphoid cells (ILC2s), and muscle ILC2s may be the main source of IL-13 [51]. IL-33 increases following exercise in circulation and induces IL-13 gene expression of immune/stromal cells in muscle [51]. Further studies are necessary to elucidate the exercise conditions inducing IL-13.

1.2.5. Histamine

Histamine is a common mediator regulating allergic reactions, anaphylactic responses, gastric acid secretion, and inflammation [53]. Histamine is mainly stored in mast cells and basophils and released by degranulation [54]. Exercise increases plasma

histamine concentration with exercise-induced anaphylaxis [55]. Exercise also increases serum histamine concentration without exercise-induced anaphylaxis [56]. However, some studies have reported that exercise did not increase plasma histamine concentration in healthy humans [57,58]. Although exercise does not dramatically induce systemic histamine response, some studies have reported that exercise induces histamine production in muscle [59,60]. For example, Romero et al. and Mungum et al. showed that exercise and heat stress increase intramuscular histamine concentrations [59,60]. Furthermore, exercise increases muscle histidine decarboxylase (HDC, the enzyme that synthesizes histamine from L-histidine) activity and gene expression [61–64].

The physiological roles of histamine in exercise are inferred from the studies of HDC knockout (KO) mice and pharmacological blockade of histamine receptors and HDC. Pharmacological blockade of both histamine H1 and H2 receptors reduces exercise capacity [65]. Furthermore, pharmacological blockade of only histamine H1 receptor or H1 receptor KO mice also reduces endurance capacity, but not blockade of H2, H3, and H4 receptors [62,63]. Pharmacological blockade of HDC and HDC KO mice also reduces endurance capacity [62,63]. These studies suggest that histamine is important for endurance capacity via H1 receptor. In addition, pharmacological blockade of histamine

H1 and H2 receptors alter transcriptome after a single bout of exercise [66] and diminishes exercise adaptation, such as increasing aerobic performance, mitochondrial function, insulin sensitivity, and capillaries in human muscle [67]. Also, HDC KO mice delay muscle regeneration [68]. These studies suggest that exercise-induced histamine response is necessary for exercise adaptation. Furthermore, pharmacological blockade of H1 and H2 receptors augments exercise-induced muscle damage, attenuates the decline of muscle strength, and reduces muscle soreness post-exercise [69].

Although the source of histamine during exercise is unidentified, one is mast cells. Exercise intramuscular histamine concentrations increased after endurance exercise with mast cell degranulation [59]. Furthermore, muscle cells are the other source of histamine because exercise increases HDC expression in muscle cells [64]. Further studies are necessary to elucidate what exercise stress induces histamine production and how histamine contributes to exercise adaptations.

1.2.6. Calprotectin

Calprotectin is a heterodimer of S100A8 and S100A9 protein, which is contained in granulocytes and monocytes [70,71]. Calprotectin increases in circulation and urine following exercise [13,15,72–75] but not eccentric exercise [76]. S100A8 and S100A9 gene

expression increases in muscle following endurance exercise [74,77]. In particular, muscle S100A8 and S100A9 gene expression levels are induced by IL-6 [74]. Furthermore, because calprotectin is released from exercising muscle [74], calprotectin is identified as a myokine. However, the roles of calprotectin for health-promoting effects are not revealed. Calprotectin modulates cytokine secretion, cellular proliferation, and antimicrobial function [71]. Furthermore, calprotectin is a ligand for toll-like receptor 4 (TLR4) and acts as endogenous damage-associated molecular patterns [70,71]. Recently, it has been revealed that muscle expressed-TLR4 is necessary for exercise adaptation, such as enhancing fatty acid, glucose oxidation, and molecular adaptations [78,79]. These studies suggest that TLR4 ligands may be necessary for exercise adaptation. Besides calprotectin, many TLR4 ligands are elevated by exercise (e.g., fatty acids, lipopolysaccharide, heat shock protein 70) [33,80,81]. Therefore, these TLR4 ligands may also induce exercise adaptations through TLR4.

1.2.7. Extracellular Vesicles

EVs are small particles secreted by various types of cells and have heterogeneity [82]. In particular, the subtypes of EV are classified based on particle size; 30-150 nm of particles are classified as exosomes, 100-1000 nm of particles are classified as

microvesicles, and > 1 μm of particles are classified as apoptotic bodies [82]. These EVs contain proteins, lipids, and nucleic acids (e.g., mRNA, microRNA (miRNA), and long non-coding RNA) and act as intercellular mediators like hormones [9,82].

Recently, EVs have been identified as a novel exerkine. In particular, 30-150 nm size EVs in circulation increased after a single bout of exercise [83,84], and exercise changes the profile of proteins, miRNAs, and mRNAs in circulating EVs [84–88]. Exercise training also changes the miRNA profile [89–91]. One source of exercise-induced EVs is muscle [84]. However, the other source is unknown. Recently, exercise-induced EVs' roles in health-promoting effects have been revealed. For example, exercise-induced EVs promote lipolysis in adipose tissue [88], inhibit anxiety [91], and prevent myocardial ischemia/reperfusion injury [89]. However, it does not elucidate which factors (e.g., exercise intensity, exercise duration, exercise mode, metabolic demand, and muscle damage) determine the quality and quantity of exercise-induced EVs and the influence of exercise-induced EVs on health-promoting effects. Further studies are necessary to elucidate what exercise stress determines the quality and quantity of exercise-induced EVs and the physiological roles of exercise-induced EVs.

1.3. The Purpose of the Studies Related to this Doctoral Thesis

As reviewed above, exerkines and their receptors are important for exercise-induced health-promoting effects. However, there are some problems with exerkine study. (1) Although many novel exerkines have been identified previously, the study of their physiological role is insufficient. (2) The studies of exerkine receptors are insufficient. (3) It is necessary to search for novel exerkine through the development of analytical technology. (4) The identification of exerkine-secreting organs/cells is difficult. Then, this thesis consists of three studies to resolve the problem as described below; (2) The studies of exerkine receptors are insufficient, and (3) It is necessary to search for novel exerkine through the development of analytical technology. The purpose of each study is shown below.

1 To elucidate the physiological roles of CCR2, a major receptor for an exerkine MCP-1, on endurance exercise.

2 To elucidate the effects of exercise training on miRNA profile in muscle-derived EVs (M-EVs).

3 To elucidate the effects of acute exercise on heparin-binding epidermal growth factor-like growth factor (HB-EGF) and amphiregulin (Areg), which are one of the humoral factors, in each organ.

Chapter 2. Pharmacological Inhibition of CCR2 Signaling Exacerbates Exercise-Induced Inflammation Independently of Neutrophil Infiltration and Oxidative Stress in Mice

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2.1. Introduction

Regular physical activity has preventative effects against various diseases (e.g., type 2 diabetes, stroke, cancer, and dementia) [1,9]. A single bout of exercise also benefits systemic health (e.g., stimulation of glucose uptake independently of insulin signaling, improvement of insulin sensitivity, and cognitive function) [3,4]. Recent research has shown that exercise-induced humoral factors known as exerkines contribute to these exercise effects [9]. However, the role of most exerkines has not been investigated. Therefore, it is important to elucidate the role of each exerkine.

Strenuous exercise induces systemic inflammation and increases the circulating concentrations of various inflammatory mediators [10,11,13,15,81]. Among the

inflammatory mediators such as cytokines and chemokines whose circulating concentrations fluctuate by exercise, MCP-1 is a representative chemokine whose concentrations increase following exercise in circulation, urine, and muscles [10–13,15,28,29,81]. MCP-1 is also secreted from C2C12 myotubes upon electric pulse stimulation, which induces muscle contraction and mimics exercise [42,43,92]. These studies suggest that MCP-1 is one of the exerkines.

MCP-1 exerts chemotactic activity for monocytes/macrophages that remove and repair damaged muscle fibers [44,45]. Following exercise, it has been suggested that MCP-1 is an important mediator for macrophage infiltration in muscle, which contributes to muscle damage and inflammation [12,28,29]. MCP-1 is a ligand for CCR2, which is expressed on monocytes/macrophages [44,45]. Several studies have reported that in various muscle injury models, CCR2 is essential for macrophage accumulation in muscle [44,45,93]. Therefore, CCR2 signaling may also be an important factor for exercise-induced macrophage infiltration and inflammation. CCR2 is also expressed on muscle cells, myogenic progenitor cells, and satellite cells, and CCR2 signaling contributes to muscle insulin resistance and dysfunction of myogenic progenitor cells [93–96]. Therefore, muscle cells may also be other targets for CCR2 ligands. However, the role of CCR2 signaling in endurance exercise has not been investigated.

Understanding the molecular mechanisms of exercise-induced inflammation can be helpful in developing appropriate countermeasures (e.g., functional food intake, meal intervention, and pharmacological approaches). Then, the present study hypothesized that CCR2 signaling is essential for macrophage accumulation and regulation of inflammation following exercise. To confirm this hypothesis, the present study administrated CCR2 antagonist to inhibit CCR2 signaling before and after exercise.

2.2. Materials and Methods

2.2.1. Animals

This study used male C57BL/6J mice (aged 10–11 weeks) provided by Takasugi Experimental Animals Supply (Kasukabe, Japan). They were housed in the breeding room under a 12-h light/dark cycle. Food (MF, oriental yeast, Tokyo, Japan) and water were freely available to all the mice. This study was authorized by the Academic Research Ethical Review Committee of Waseda University (2020-A29).

2.2.2. Experimental Protocols

To investigate the role of CCR2 signaling 24 h after exercise, the mice were

randomly divided into four groups as follows: rest with phosphate-buffer saline (PBS) administration, rest with CCR2 antagonist administration, exercise with PBS administration and exercise with CCR2 antagonist administration. The CCR2 antagonist RS-504393 (Abcam, Cambridge, U.K.) was dissolved in PBS (100 μg in 100 μL of PBS; approximately 4 mg/kg body weight), or 100 μL of PBS was administered orally 1 h before and 12 h after the first administration. The exercised mice were sacrificed 24 h after the exercise, and the rest mice were sacrificed in the same time course as the exercised mice (n = 6, per group).

To investigate the role of CCR2 signaling immediately after exercise, mice were randomly divided into four groups in the same manner. Dissolved RS-504393 (100 μg in 100 μL of PBS; approximately 4 mg/kg body weight) or 100 μL of PBS was administered orally 1 h before exercise. The exercised mice were sacrificed immediately after the exercise, and the rest mice were sacrificed in the same time course as the exercise mice (n = 6, per group).

The following exercise protocol was performed as previously described with minor modification [12]. A detailed protocol is described below. Four days before the experiments, all mice were accustomed to treadmill running at 15 m/min and 7% grade for 10 min. On the experimental days, the mice were subjected to treadmill running at

24 m/min and 7% grade for 60 min. Heparinized blood samples were collected from the abdominal aorta under isoflurane inhalation anesthesia (Abbott, Tokyo, Japan), and then the mice were perfused by PBS to remove blood in the tissues. After cervical dislocation, the gastrocnemius, kidney, liver, and epididymal adipose tissues were harvested and frozen in liquid nitrogen. Blood samples were centrifuged at 1600 g for 10 min at 4°C to extract plasma samples. All samples were maintained at −80°C.

2.2.3. Real-time PCR

This study performed real-time PCR for mRNA expression as previously described [12], and a detailed protocol is described below. Total RNA was extracted from the gastrocnemius, kidney, liver, and adipose tissues using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentrations of the extracted total RNA were measured using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reaction (PCR) was performed using the Fast 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) and Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The PCR condition was below; denaturing at 95°C

for 20 s followed by 40 cycles of denaturing at 95°C for 3 s, and annealing and elongation at 60°C for 30 s. Each gene was normalized using 18s ribosomal RNA. Because the difference in kidney ß-actin expression between groups was smaller than 18s ribosomal RNA expression (data not shown), this study used ß-actin for the normalization of kidney gene expression. Although 18s ribosomal RNA expression was significantly different between groups in some tissues, the maximal fold change of each housekeeping gene expression between groups was 1.3 times (data not shown). Therefore, this study concluded that the effects of normalization using 18s ribosomal RNA or ß-actin are small. All data were calculated using the ΔΔCt method. The specific primer sequences are shown in Table 1.

Table 1. Primer sequences for real time-PCR.

	Forward	Reverse
18s	TTCTGGCCAACGGTCTAGACAAC	CCAGTGGTCTTGGTGTGCTGA
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
ATF4	AACCTCATGGGTTCTCCAGCGA	CTCCAACATCCAATCTGTCCCG
β-actin	GCGGACTGTTACTGAGCTGCGT	TGCTGTCGCCTTCACCGTTCC
CAT	ACATGGTCTGGGACTTCTGG	CAAGTTTTTGATGCCCTGGT
CCR2	ACAGCTCAGGATTAACAGGGACTTG	ACCACTTGCATCCACACATGAC
CCR5	CATCCGTTCCCCCTACAAGA	GGAACTGACCCTTGAAAATCCA
CD11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
CD163	GGGTCATTCAGAGGCACACTG	CTGGCTGTCCTGTCAAGGCT
CD206	CAAGGAAGGTTGGCATTTGT	CCTTTCAGTCCTTTGCAAGC
CD68	CTTCCCACAGGCAGCACAG	AATGATGAGAGGCAGCAAGAGG
CHOP	TATCTCATCCCCAGGAAACG	TATCTCATCCCCAGGAAACG
CX3CL1	ACGAAATGCGAAATCATGTGC	CTGTGTCGTCTCCAGGACAA
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
GSTm3	GCTCTTACCACGTGCAGCTT	GGCTGGGAAGAGGAAATGGA

HO-1	CACGCATATACCCGCTACCT	CCAGAGTGTTCATTCGAGCA
IL-10	CGCAGCTCTAGGAGCATGTG	GCTCTTACTGACTGGCATGAG
IL-1β	GGGCCTCAAAGGAAAGAATC	TTGCTTGGGATCCACACTCT
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-6	AACGATGATGCACTTGCAGA	TGGTACTCCAGAAGACCAGAGG
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
MCP-2	AGAGACAGCCAAAGCTGGAA	CAGGCACCATCTGCTTGTAA
MCP-3	CACATTCCTACAGACAGCTC	AGCTACAGAAGGATCACCAG
MIP-1α	ACTGCCTGCTGCTTCTCCTACA	ATGACACCTGGCTGGGAGCAAA
MIP-1β	ACCCTCCCACTTCCTGCTGTTT	CTGTCTGCCTCTTTTGGTCAGG
NADPH Oxidase	TTGGGTCAGCACTGGCTCTG	TGGCGGTGTGCAGTGCTATC
NQO1	GGTATTACGATCCTCCCTCAACATC	GAGTACCTCCCATCCTCTCTTCTTC
Nrf1	GTGGGACAGCAAGCGATTGTAC	CGCACCACATTCTCCAAAGG
Nrf2	CTCGCTGGAAAAAGAAGTGG	CCGTCCAGGAGTTCAGAGAG
ORP150	CAGACTGAAGAGGCGAAACC	TTCCTGTTCAGGTCCAGCTC
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Sirt1	GCAACAGCATCTTGCCTGAT	GTGCTACTGGTCTCACTT
SOD1	GAGACCTGGGCAATGTGACT	GTTTACTGCGCAATCCCAAT
SOD2	TCAAGCGTGACTTTGGGTCT	AGCGGAATAAGGCCTGTTGT
TNF-α	CCTCCCTCTCATCAGTTCTA	ACTTGGTGGTTTGCTACGAC

Arg1, Arginase1; ATF4, activating transcription factor 4; CAT, catalase; CCR, CC chemokine receptor; CHOP, C/EBP homologous protein; GST, glutathione S-transferase; HO-1, heme oxygenase-1; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; NQO, NADPH quinone dehydrogenase; Nrf, NF-E2-related factor; ORP150, oxygen-regulated protein 150; PGC-1α, peroxisome proliferator-activated receptor coactivator-1α; Sirt1, sirtuin 1; SOD, superoxide dismutase; TNF, tumor necrosis factor.

2.2.4. Histological Analysis

Cross sections of 10 μm frozen gastrocnemius were used for hematoxylin and eosin (H&E) staining and immunohistochemistry. For immunohistochemistry, the sections were first fixed in 4% paraformaldehyde for 7 min and then incubated with 5% bovine serum albumin (BSA) in PBS for 30 min. Next, the sections were incubated overnight at 4°C with primary antibodies in PBS with 5% BSA. After washing the sections, they were incubated with the secondary antibodies in PBS with 5% BSA for 1

h at room temperature. After washing, the sections were mounted using the Vectashield mounting medium (H-1800, Vector Laboratories, Burlington, ON, Canada). The antibodies used in this study are shown in Table 2. The stained sections were observed under a fluorescence microscope BZ-8000 (KEYENCE, Osaka, Japan). Three fields at ×200 magnification were captured per animal to identify the localization of IL-6. The number of M1 and M2 macrophages was quantified according to the previous study [97]. Briefly, this study defined M1 macrophages as $F4/80^+$ $CD206^-$ cells and M2 macrophages as $F4/80^+$ $CD206^+$ cells in the same section. Two to four fields at ×100 magnification were captured per animal to quantify the number of macrophages. This study then calculated the average value of each field as the measured data.

Table 2. Antibodies for immunohistochemistry.

Antibodies	Source	Identifier	Dilution (Concentration)
Goat anti-IL-6 antibody	R&D Systems, Minneapolis, MN, USA	Cat# AF406	10 μg/mL
Mouse anti-dystrophin antibody (clone 1808)	Abcam, Cambridge, U.K.	Cat# ab3149	1:400
Rat anti-F4/80 antibody (clone BM8)	Biolegend, San Diego, CA, USA	Cat# 123101	1:100
Goat anti-CD206 antibody	R&D Systems, Minneapolis, MN, USA	Cat# AF2535	2 μg/mL
Alexa Fluor 555-conjugated rabbit anti-goat IgG antibody	Thermo Fisher Scientific, Waltham, MA, USA	Cat# A21431	1:200
Fluorescein-conjugate horse anti-mouse IgG antibody	Vector Laboratories, Burlington, ON, Canada	Cat# FI-2000	1:200
Alexa Fluor 488-conjugated donkey anti-rat IgG antibody	Thermo Fisher Scientific, Waltham, MA, USA	Cat# A21208	1:200
Alexa Fluor 555-conjugated donkey anti-goat IgG antibody	Abcam, Cambridge, U.K.	Cat# ab150130	1:200

2.2.5. ELISA and TBARS Assay

Plasma and tissue MCP-1 concentrations were measured using the Mouse MCP-1 DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA), tissue IL-1β concentrations were measured using the Mouse IL-1β DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA), and tissue myeloperoxidase (MPO) concentrations were measured using the MPO ELISA kit (Hycult Biotech, Uden, The Netherlands). Gastrocnemius thiobarbituric acid reactive substance (TBARS) concentrations were measured using the TBARS assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). To measure the tissue concentrations of MCP-1, IL-1β, MPO, and TBARS, at first, this study extracted tissue proteins as previously described [12]. The tissue was homogenized in the tissue protein extraction reagent (T-PER; Thermo Fisher Scientific, Waltham, MA, USA) containing a protease inhibitor (complete mini protease inhibitor cocktail tablet; Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at 10,000 g for 15 min at 4°C and the supernatant was collected. To avoid tissue debris contamination, the supernatant was centrifuged again. Total protein concentrations in the supernatant were measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was used for each assay. The tissue concentrations of MCP-1, IL-1β, MPO, and TBARS were normalized using the total protein in the supernatant. The absorbance

was measured using Spectra Max iD5 (Molecular Devices Inc., San Jose, CA, USA).

2.2.6. Statistical Analysis

Data are expressed as mean ± standard error (SE). Two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used for analyzing data. Statistical analysis was performed using SPSS V25.0 (IBM Japan, Ltd., Tokyo, Japan), with statistical significance being defined as $p < 0.05$.

2.3. Results

2.3.1. Inhibition of CCR2 Signaling Exacerbates Macrophage Infiltration and Inflammation 24 h after Exercise in Muscle

The previous studies reported that exercise induces macrophage infiltration in muscle, which contributes to exercise-induced inflammation [12,28,29,97]. The previous studies observed macrophage infiltration 24 h after exercise [28,29,97]. To investigate the role of CCR2 signaling in exercise-induced macrophage infiltration and inflammation in muscle, this study administrated CCR2 antagonist to inhibit CCR2 signaling before and after exercise, and the gastrocnemius was excised 24 h after exercise. In contrast to the expectation, exercise with CCR2 antagonist administration induced the gene

expression of a pan-macrophage marker (F4/80), M1 macrophage markers (CD68, CD11c), an M2 macrophage marker (arginase 1), and chemokine receptors expressed on M1 macrophages (CCR2, CCR5) [44,93,98] (Figure 1A). The histological analysis revealed that exercise with CCR2 antagonist administration promoted the infiltration of M1 macrophages ($F4/80^+$ $CD206^-$ cells) (Figure 1B,C). However, exercise with PBS administration did not induce such changes (Figure 1A–C). The histological analysis also revealed that the infiltration of M2 macrophages ($F4/80^+$ $CD206^+$ cells) did not change after exercise with and without CCR2 antagonist administration (Figure 1B,D). Muscle injury induces immune cell infiltration and inflammation [44,45,93,97]. However, this study did not observe abnormal muscle fibers (e.g., necrotic fibers) in all groups (Figure 1E). M1 macrophages produce inflammatory cytokines and chemokines [44]. This study next investigated the local inflammation. Exercise with CCR2 antagonist administration induced the gene expression of cytokines and chemokines such as IL-1β, TNF-α, IL-10, MCP-1, MCP-3, and macrophage inflammatory protein-1β (MIP-1β) (Figure 1F). However, exercise with PBS administration did not induce such changes (Figure 1F). This study next measured the concentrations of plasma MCP-1 to investigate whether inhibition of CCR2 signaling exacerbates systemic inflammation. However, plasma MCP-1 concentrations showed no changes after exercise with and without CCR2

antagonist administration (Figure 1G). These results indicated that the inhibition of CCR2 signaling exacerbated exercise-induced M1 macrophage infiltration and inflammation at a local level 24 h after exercise.

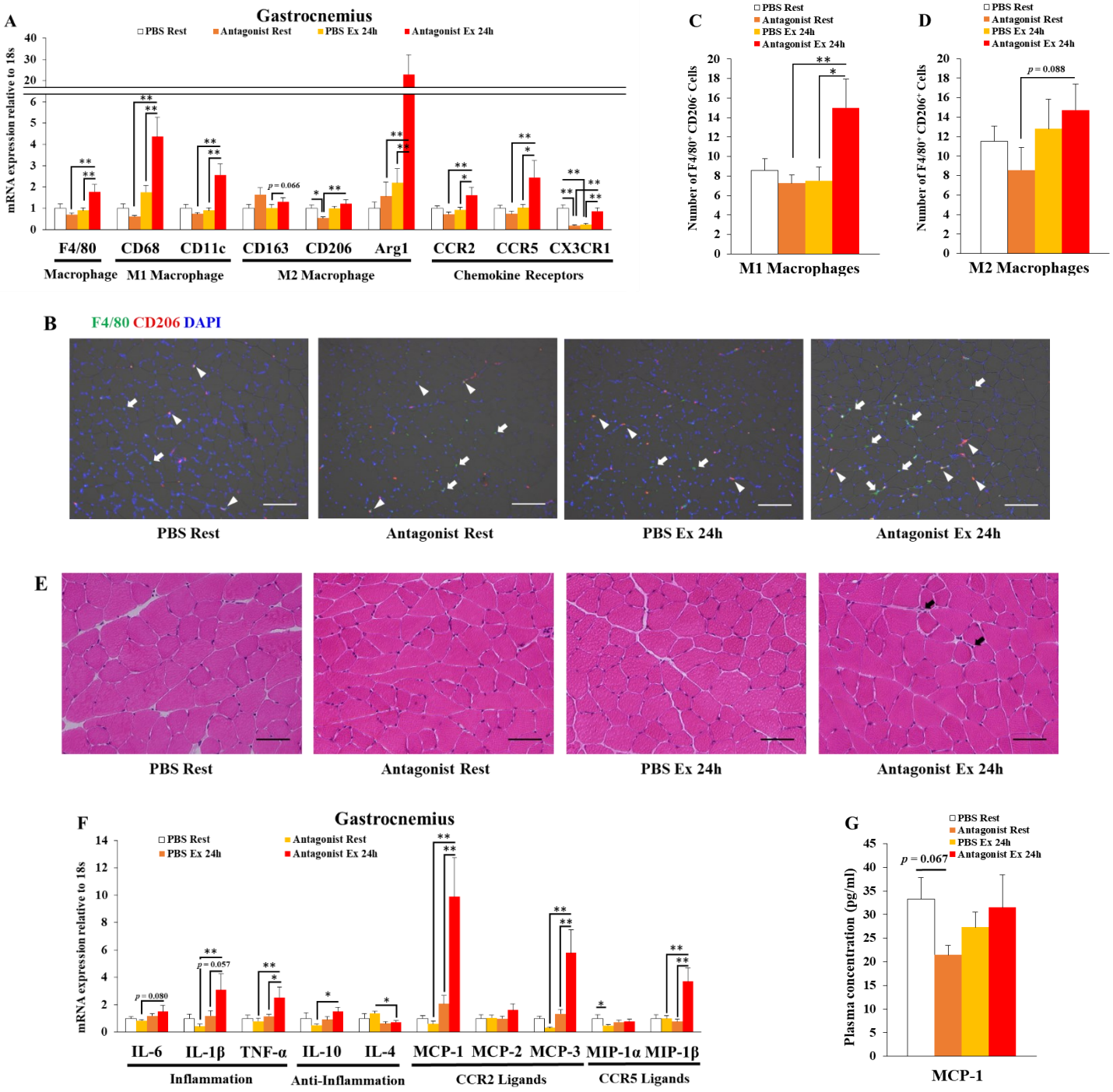


Figure 1. (A) Gene expression of macrophage markers (n = 5–6). (B) Representative images of gastrocnemius sections using immunofluorescence staining (green, F4/80; red, CD206; blue, DAPI; magnification ×100, n = 5–6). Images were merged with bright field. Arrows indicate M1 macrophages ($F4/80^+$ $CD206^-$ cells, n = 5–6). Arrowheads indicate M2 macrophages ($F4/80^+$ $CD206^+$ cells, n = 5–6). Scale bar is 100 μm. (C) The number of M1 macrophages per field. (D) The number of M2 macrophages per field. (E) Representative images of gastrocnemius sections using H&E staining (magnification ×200, n = 3). Arrowheads indicate immune cell infiltration. Scale bar is 50 μm. (F) Gene expression of inflammation (n = 5–6). (G) Plasma MCP-1 concentrations (n = 5–6). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [* $p < 0.05$, ** $p < 0.01$]. Arg1, Arginase1; CCR, CC chemokine receptor; IL, interleukin; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; ANOVA, analysis of variance; SE, standard error.

2.3.2. Inhibition of CCR2 Signaling Exacerbates Exercise-induced Inflammation Immediately after Exercise Independently of Neutrophil Infiltration in Muscle

This study next investigated the role of CCR2 signaling in inflammation immediately after exercise in muscle. Exercise with CCR2 antagonist administration induced the gene expression of cytokines and chemokines such as IL-6, IL-1β, TNF-α, IL-10, MCP-3, and MIP-1β, but not the macrophage marker, F4/80 (Figure 2A). Exercise with CCR2 antagonist administration also induced the increase in IL-1β protein concentrations (Figure 2B). However, exercise with PBS administration did not induce such changes (Figure 2A,B). Moreover, plasma MCP-1 concentrations, as an indicator of systemic inflammation, showed no changes after exercise (Figure 2C). These results indicated that the inhibition of CCR2 signaling exacerbated exercise-induced inflammation at a local level immediately after exercise. This study also did not observe

necrotic muscle fibers in all groups (Figure 2D), which suggested that necrosis of muscle fibers does not cause this inflammation.

Kawanishi, et al. have previously reported that neutrophils contribute to exercise-induced inflammation in muscle [29]. Therefore, this study hypothesized that neutrophil infiltration contributes to exercise-induced inflammation by the inhibition of CCR2 signaling. This study found that exercise with and without CCR2 antagonist administration increased the concentrations of gastrocnemius MPO, a neutrophil infiltration marker (Figure 2E). However, the gastrocnemius MPO concentrations showed no difference between exercise with and without CCR2 antagonist administration (Figure 2E). This result suggested that CCR2 signaling inhibition exacerbated exercise-induced inflammation independently of neutrophil infiltration.

This study next investigated the localization of IL-6 to identify the source of inflammatory cytokine. The histological analysis revealed that IL-6 is primarily localized in the interfiber space (Figure 2F). However, exercise with CCR2 antagonist administration also induced IL-6 expression in muscle fibers, but the signal was weak compared to that in the interfiber space (Figure 2F). This result suggested that muscle fibers may contribute to exercise-induced inflammation by inhibition of CCR2 signaling.

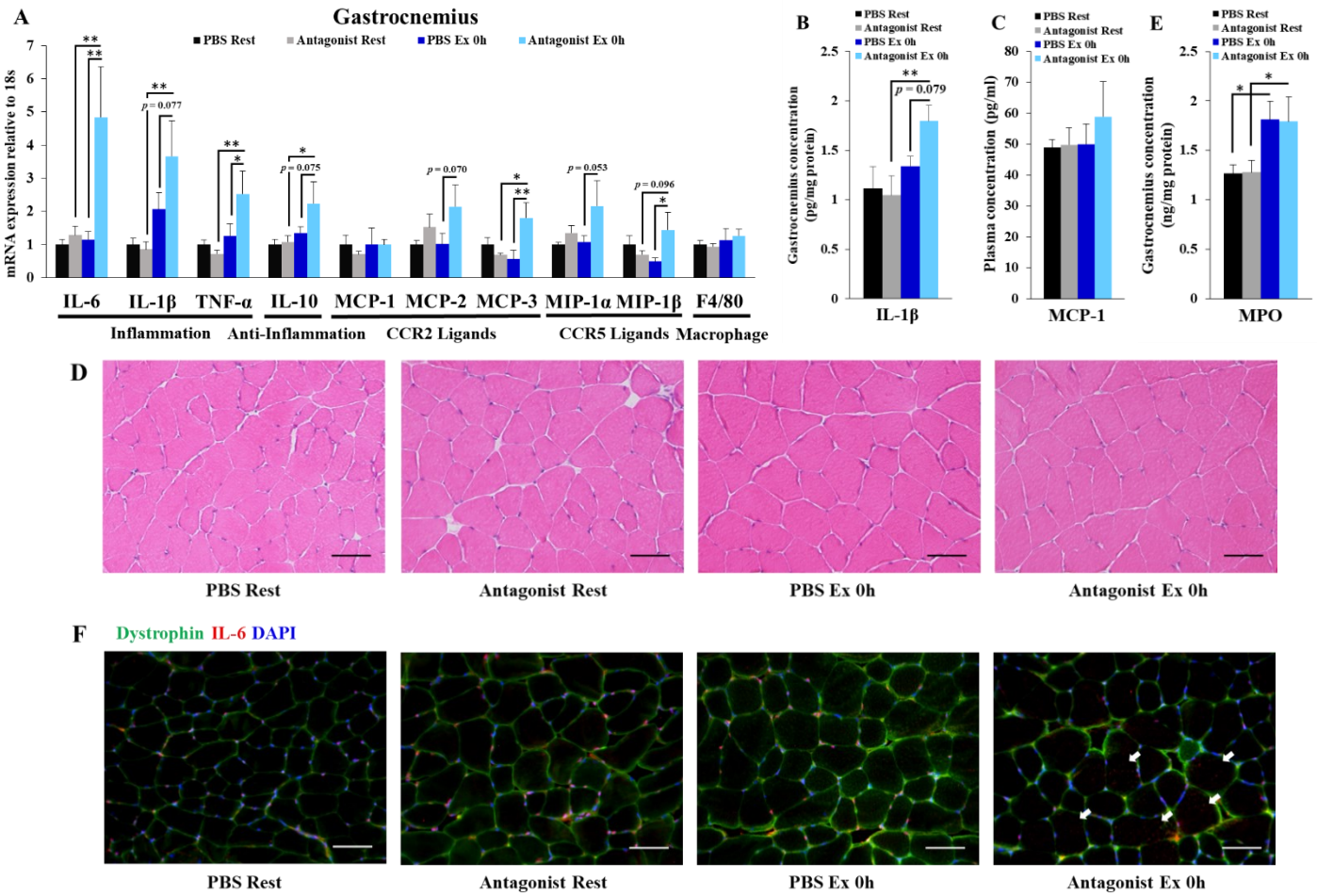


Figure 2. (A) Gene expression of inflammation (n = 5–6). (B) Gastrocnemius IL-1ß concentrations (n = 6) (C) Representative images of gastrocnemius sections using immunofluorescence staining (green, dystrophin; red, IL-6; blue, DAPI; magnification ×200, n = 2). Arrowheads indicate IL-6 positive muscle fibers. Scale bar is 50 μm. (D) Representative images of gastrocnemius sections using H&E staining (magnification ×200, n = 2). Scale bar is 50 μm. (E) Plasma MCP-1 concentrations (n = 5–6). (F) Gastrocnemius MPO concentrations (n = 6). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [* $p < 0.05$, ** $p < 0.01$]. IL, interleukin; TNF-α, tumor necrosis factor-α; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; ANOVA, analysis of variance; SE, standard error.

2.3.3. Inhibition of CCR2 Signaling Does Not Influence Muscle Oxidative Stress Immediately after Exercise

Oxidative stress is one of the triggers of exercise-induced inflammation [81].

Hence, this study next hypothesized that oxidative stress contributes to exercise-induced inflammation by the inhibition of CCR2 signaling. However, this study observed that there were no changes in the concentrations of gastrocnemius TBARS, an oxidative stress marker, by exercise with and without CCR2 antagonist administration (Figure 3A). Moreover, the gene expression of the oxidative enzyme NADPH oxidase showed no changes (Figure 3B). These results indicated that oxidative stress does not contribute to exercise-induced inflammation by the inhibition of CCR2 signaling. Because NF-E2-related factor 2 (Nrf2) is known to activate anti-inflammatory systems [99,100], the present study hypothesized that Nrf2 is an important factor of exercise-induced inflammation by inhibiting CCR2 signaling. Nrf2 gene expression was increased by exercise with and without CCR2 antagonist administration (Figure 3B). However, the downstream genes of Nrf2 such as heme oxygenase-1 (HO-1), glutathione S-transferase (GST), and NADPH quinone dehydrogenase-1 (NQO-1) [99,100] exhibited inconsistent changes (Figure 3B). These results indicated that the Nrf2 pathway did not activate and may not contribute to exercise-induced inflammation by the inhibition of CCR2 signaling.

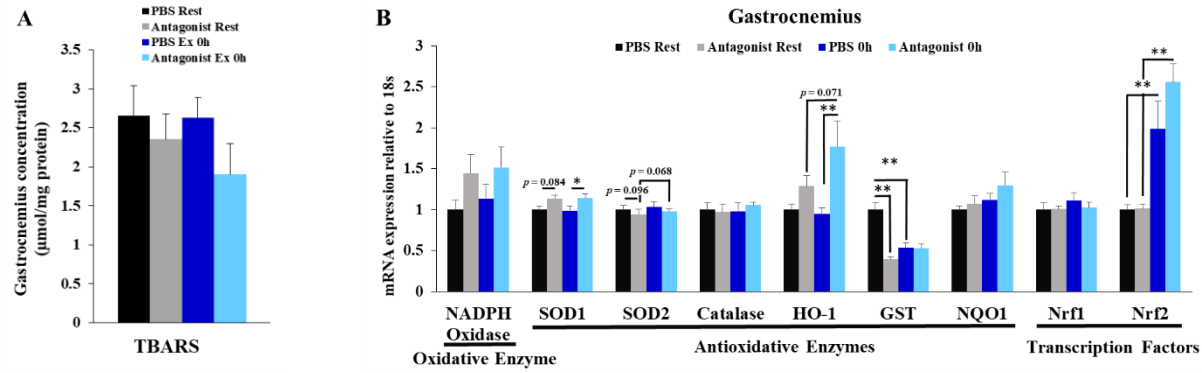


Figure 3. (A) Gastrocnemius TBARS concentrations (n = 6). (B) Gene expression of an oxidative enzyme, antioxidative enzymes and transcription factors (n = 6). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [* $p < 0.05$, ** $p < 0.01$]. TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; HO-1, heme oxygenase-1; GST, glutathione S-transferase; NQO, NADPH quinone dehydrogenase; Nrf, NF-E2-related factor; ANOVA, analysis of variance; SE, standard error.

2.3.4. Effects of CCR2 Signaling Inhibition on Muscle Gene Expression of ER Stress Marker and Exercise Adaptation-related Genes Immediately after Exercise

Endoplasmic reticulum (ER) stress is known to induce inflammation [101]; therefore, this study next hypothesized that ER stress is an important factor of exercise-induced inflammation by inhibiting CCR2 signaling. The gene expression of the ER stress marker oxygen-regulated protein 150 (ORP150) was induced by exercise with CCR2 antagonist administration (Figure 4). However, the expression of other markers activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) did not increase by exercise (Figure 4). These results indicated that ER stress may not strongly contribute to exercise-induced inflammation by the inhibition of CCR2 signaling. The

expression of exercise adaptation-related genes such as peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) and sirtuin 1 (Sirt1) [2] also did not increase by exercise (Figure 4).

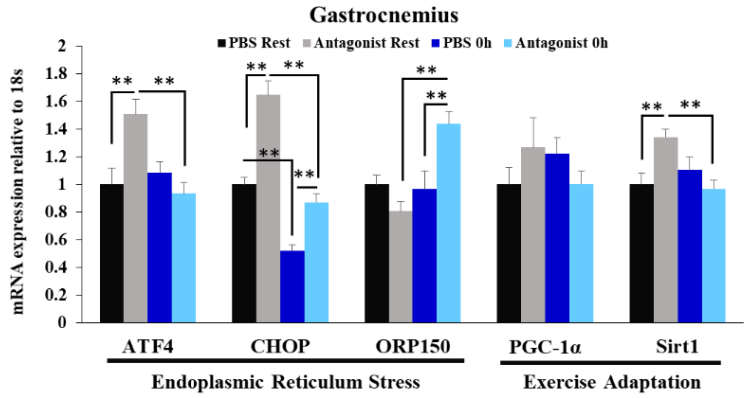
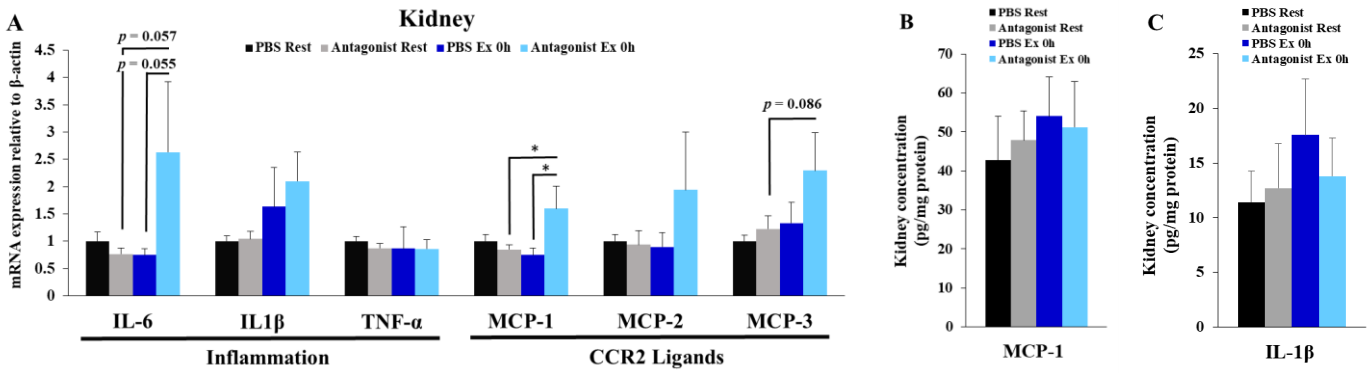


Figure 4. Gene expression of endoplasmic reticulum stress marker and inducing exercise adaptation (n = 6). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [** $p < 0.01$]. ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; ORP150, oxygen-regulated protein 150; PGC-1α, peroxisome proliferator-activated receptor coactivator-1α; Sirt1, sirtuin 1; ANOVA, analysis of variance; SE, standard error.

2.3.5. Inhibition of CCR2 Signaling Exacerbates Exercise-induced Inflammation in Kidney, Liver, and Adipose Tissues

It is known that strenuous exercise induces internal organ damage and inflammation [81]. Hence, this study next investigated the effects of CCR2 signaling inhibition on exercise-induced inflammation in kidney, liver, and adipose tissues. In the kidney, exercise with CCR2 antagonist administration induced MCP-1 gene expression

but did not change MCP-1 protein concentrations (Figure 5A,B). Kidney IL-1β concentrations also showed no changes after exercise (Figure 5C). In the liver, exercise with CCR2 antagonist administration induced the gene expression of IL-6, IL-1β, MCP-1, and MCP-2, but it did not change MCP-1 protein concentrations (Figure 5D,E). The concentrations of IL-1β in the liver showed an increasing trend after exercise with CCR2 antagonist administration (Figure 5F). In the adipose tissue, exercise with CCR2 antagonist administration induced MCP-3 gene expression but did not change the concentrations of MCP-1 and IL-1β proteins (Figure 5G–I). These results indicated that the inhibition of CCR2 signaling exacerbated exercise-induced inflammation in the kidney, liver, and adipose tissues.



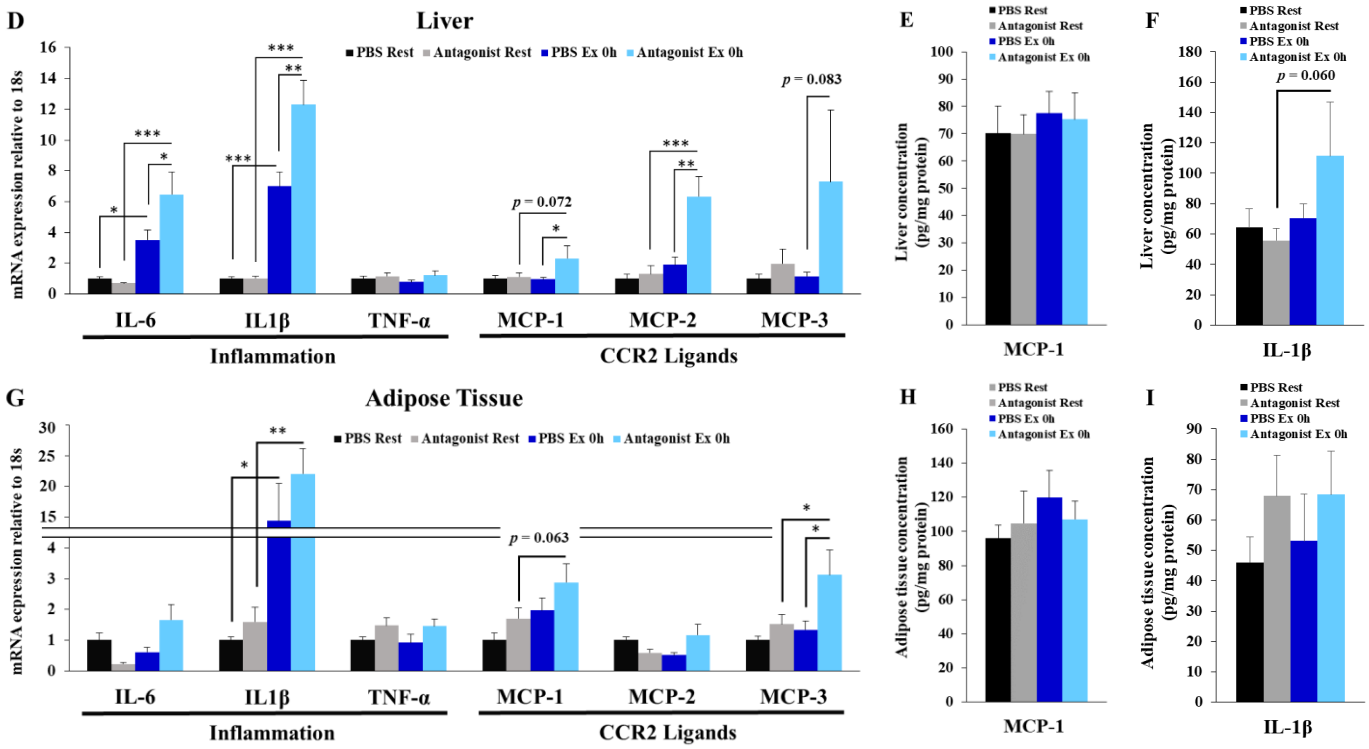


Figure 5. Gene expression of inflammation in (A) kidney, (D) liver and (G) adipose tissues (n = 6, in each tissue). MCP-1 concentrations in (B) kidney, (E) liver and (H) adipose tissues (n = 6, in each tissue). IL-1β concentrations in (C) kidney, (F) liver and (I) adipose tissues (n = 6, in each tissue). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$]. IL, interleukin; TNF-α, tumor necrosis factor-α; MCP, monocyte chemoattractant protein; ANOVA, analysis of variance; SE, standard error.

2.3.6. CCR2 Ligand-producing Organs

Although the inhibition of CCR2 signaling exacerbates exercise-induced inflammation, the mechanisms underlying this phenomenon are unidentified. Therefore, this study hypothesized that increasing concentrations of CCR2 ligands in circulation regulate exercise-induced inflammation. This study then focused on plasma MCP-1 concentrations before and immediately after exercise in the PBS-administrated group.

However, this study observed no changes in plasma MCP-1 concentrations after exercise in the PBS-administrated group (Figure 2C). This study next hypothesized that tissue CCR2 ligands act in an autocrine manner. However, this study found that the gene expression levels of MCP-1, MCP-2, and MCP-3 in the gastrocnemius, kidney, liver, and adipose tissues did not change after exercise in the PBS-administrated group (Figures 2A and 5A,D,G). Moreover, MCP-1 protein concentrations in the kidney, liver, and adipose tissues showed no changes after exercise in the PBS-administrated group (Figure 5B,E,H). These results indicated that increasing concentrations of MCP-1 in circulation and gastrocnemius, kidney, liver, and adipose tissues do not contribute to exercise-induced inflammation by the inhibition of CCR2 signaling. Increasing tissue concentrations of MCP-2 and MCP-3 proteins may also not contribute to exercise-induced inflammation by the inhibition of CCR2 signaling.

2.4. Discussion

In the present study, in contrast to the expectation that the inhibition of CCR2 signaling inhibits exercise-induced macrophage infiltration, the present study observed that CCR2 signaling inhibition exacerbated macrophage infiltration following exercise. This result is probably because receptors other than CCR2 mediate macrophage

infiltration. Macrophages express various chemokine receptors (e.g., CCR2, CCR5, CCR7, and CXCR1) [44,93,98,102], and several studies have reported that CCR5 and CCR7 contribute to macrophage infiltration in the adipose tissue of obese mice [98,102]. However, in the acute muscle injury model, whether CCR5 and CCR7 contribute to macrophage infiltration remains unexplored. In the present study, the gene expressions of CCR5 and CCR5 ligands, MCP-3 and MIP-1β, were increased 24 h after exercise with CCR2 antagonist administration. Therefore, CCR5 may mediate macrophage infiltration in this model. The present study did not measure CCR7 gene expression. However, CCR7 and CCR7 ligands may also mediate macrophage infiltration in this model.

The present study also found that CCR2 signaling inhibition exacerbated exercise-induced inflammation independently of neutrophil activation and oxidative stress immediately after exercise. However, a further concern is about those factors that regulate exercise-induced inflammation by the inhibition of CCR2 signaling. One hypothesis is that increasing levels of CCR2 ligands regulate exercise-induced inflammation. However, the present study did not change circulating MCP-1 concentrations, one of the CCR2 ligands. One possibility is that circulating MCP-2 or MCP-3 concentrations are important, which were not measured in the present study. Another possibility is that organs not investigated in the present study produce CCR2

ligands locally and regulate exercise-induced inflammation in the entire body. Therefore, further studies are required to identify the CCR2 ligands whose levels fluctuate following exercise in circulation and organs. Another hypothesis is that basal CCR2 signaling is essential to regulate exercise-induced inflammation. Because the activation of CCR2 signaling phosphorylates more than 200 proteins [103], inhibiting basal CCR2 signaling may also influence gene expression and protein phosphorylation. These genes or phosphorylated proteins may be essential for regulating exercise-induced inflammation. In the present study, CCR2 antagonist administration alone also regulated the gene expression of GST, ATF4, CHOP, and Sirt1. Further research is necessary to identify key factors that trigger exercise-induced inflammation by CCR2 signaling.

Eotaxin is a natural antagonist for CCR2 [104,105]. Therefore, eotaxin may contribute to exercise-induced inflammation. However, several studies have reported that circulating eotaxin concentrations showed no changes following endurance exercise [39,106], and our unpublished data revealed that eotaxin/CCL11 gene expression does not change following exhaustive exercise in the mice gastrocnemius (data not shown). In contrast, cultured myotubes with electric pulse stimulation secrete eotaxin/CCL11 [92]. Therefore, local eotaxin in each organ may contribute to exercise-induced inflammation. There is evidence showing that CCR2 antagonist administration improves

atherosclerosis and type 2 diabetes [107,108] and is expected to be an effective therapeutic agent. Nevertheless, CCR2 antagonist has not yet led to the development of medicines for the disease. The present findings may contribute to understanding the side effects of CCR2 antagonist.

The present study orally administrated the CCR2 antagonist RS-504393 to inhibit CCR2 signaling as previous research [109]. However, the present study did not explore whether this administration protocol is appropriate to inhibit CCR2 signaling. Therefore, it is important to validate whether this protocol is appropriate for CCR2 signaling (e.g., the experiment on whether RS-504393 administration inhibits the response of recombinant MCP-1 protein injection at several time points after RS-504393 administration). In addition, RS-504393 exerts an antagonizing activity against the α1-adrenergic receptor [110,111]. Exercise increases the concentrations of circulating adrenalin, which modulates immune systems [1]. Therefore, the present study may also indicate the role of the α1-adrenergic receptor. Further studies are necessary using CCR2-KO mice or neutralizing antibodies for CCR2 ligands such as MCP-1, MCP-2, and MCP-3 are necessary to investigate the role of CCR2 signaling in exercise specifically.

2.5. Conclusions

The present study investigated the role of CCR2 signaling on exercise-induced inflammation using CCR2 antagonist RS-504393. The present study showed that inhibition of CCR2 signaling exacerbated exercise-induced M1 macrophage infiltration and gene expression of inflammatory cytokines. This study elucidated a part of the role of CCR2 signaling regarding exercise. Further studies are necessary to investigate the role of CCR2 signaling, more specifically, using CCR2 KO mice.

Chapter 3. Six Weeks of Voluntary Exercise Training and High Fat Diet Feeding do not Change the miRNA Profile in Muscle-derived Extracellular Vesicles

3.1. Introduction

Exercise has various benefits for systemic conditions, such as improvement of type 2 diabetes, brain functions, preventive effects on cancer, and anti-inflammatory effects against chronic inflammation [1]. As one of the mechanisms for such health-promoting effects, exercise-induced humoral factors, called exerkine, is considered to contribute to these benefits [9]. Recently, EVs have been identified as a novel exerkine [84,112]. EVs contain proteins, lipids, miRNAs, mRNAs, and other biologically active molecules [9,82] and are secreted by various cell types and mediate intercellular/interorgan communication [9,84]. Acute endurance exercise increases the number of circulating EVs and changes the protein profile and the miRNA profile in circulating EVs [83,84,86–88,113,114]. Exercise training also changes the miRNA profile in circulating EVs [89–91]. Therefore, exercise-induced EVs may mediate health-promoting effects [9,115]. Some studies have reported the role of miRNAs in EVs modulated by exercise. For example, acute resistance exercise-induced miR-1 in EVs

promotes lipolysis in adipose tissue [88]. Furthermore, exercise training-induced miR-342 in EVs has a cardioprotective effect against myocardial ischemia/reperfusion injury [89]. Many studies have reported that exercise promotes EV secretion. However, the source of exercise-induced EVs is not fully understood. One of the sources is skeletal muscle, and such M-EVs reach remote organs (e.g., liver and adipose tissue) [84,88,112]. Even in a resting state, M-EVs are released into the circulation [116]. M-EVs may also mediate intercellular communication in intramuscular tissue in an autocrine/paracrine manner [117–119]. Therefore, it is important to investigate the characteristics of exercise-induced M-EVs.

Obesity induces chronic inflammation and increases the risk of various diseases [1]. In the pathophysiologies of obesity, numerous researchers have focused on the characteristics of EVs [120,121]. In particular, obesity changes the miRNA profile in M-EVs [122–124]. Therefore, the change of miRNA profile in M-EVs may be one of the pathophysiologies of obesity. On the other hand, exercise training improves obesity-induced chronic inflammation [1] and restores the abnormal miRNA profile in circulating EVs of obese mice normally [90]. Therefore, exercise training also may improve the abnormal miRNA profile in M-EVs induced by obesity.

Against these backgrounds, the present study hypothesized that exercise

training and obesity change the miRNA profile in M-EVs. To confirm this hypothesis, the present study had mice voluntary exercise training and fed high fat diet (HFD) for 6 weeks. Then, the present analyzed the miRNA profile in M-EVs obtained using ex vivo explant muscle culture model.

3.2. Materials and Methods

3.2.1. Animals, Voluntary Exercise Training, and Diets

Male C57BL/6J mice were provided from Takasugi Experimental Animals Supply (Kasukabe, Japan) and were bred under a 12-h light/dark cycle. This study was authorized by the Academic Research Ethical Review Committee of Waseda University (2021-A114).

The mice were randomly assigned into four groups: normal diet (ND) feeding with sedentary group (ND + Sed, n = 8), ND feeding with voluntary exercise training group (ND + Ex, n = 6), HFD feeding with sedentary group (HFD + Sed, n = 8), and HFD feeding with voluntary exercise training group (HFD + Ex, n = 6). The ND consisted of 10% fat calories, 20% protein calories, and 70% carbohydrate calories (D12450J; Research Diets, New Brunswick, NJ, USA), and the HFD consisted of 60% fat calories, 20% protein calories, and 20% carbohydrate calories (D12492; Research Diets, New

Brunswick, NJ, USA). The mice were fed the ND or HFD from 8 weeks of age for 6 weeks. Food and water were freely available to all the mice. The ND and HFD groups of mice were individually housed in cages without running wheels. The ND + Ex and HFD + Ex group of mice were individually housed in cages with running wheels starting at 8 weeks of age for 6 weeks (Running wheels; RWC-15, Melquest, Toyama, Japan). The number of rotations was recorded every day. The ND + Ex and HFD + Ex group mice were moved in the cages without running wheels 2 days before sacrifice to avoid the effects of acute exercise. All the mice fasted for 16-20 h before sacrifice. One gastrocnemius was used for isolating M-EVs, and the other gastrocnemius was quickly frozen in liquid nitrogen and used for real-time PCR.

3.2.2. EV Isolation

M-EVs were isolated as described previously with minor modifications [116,117,119,122–125]. Excised gastrocnemius was cut into small pieces in 4.5 g/L glucose-containing serum-free DMEM and cultured for 24 h in a 95% O_2/5% CO_2-supplemented incubator at 37°C. To remove cellular debris, the cultured medium was centrifuged at 10,000 g for 30 min. The supernatant was then filtered using a 0.22 μm filter. EVs were isolated from the cultured medium using Total Exosome Isolation

Reagent from cell culture media (Thermo Fisher Scientific, Waltham, MA, USA). Isolated EVs were resuspended in PBS and kept at -80°C until further analysis.

3.2.3. Western Blotting

Three random M-EV samples were picked up and used for western blotting. The M-EVs protein concentrations were measured using a Qubit™ protein assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The samples were mixed with 4 × Laemmli sample buffer (Bio-Rad, Richmond, CA, USA) in 10% 2-mercaptoethanol and boiled at 95°C for 5 min. A total 50 μg of protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 7.5% acrylamide-bis gel and then transferred to PVDF membrane. The transferred PVDF membrane was stained by Ponceau S to check equal loading and transfer. After that, the PVDF membrane was incubated with PVDF Blocking Reagent (TOYOBO, Osaka, Japan) for blocking. Then, the membrane was incubated overnight at 4°C with mouse anti-Alix antibody (1:1000, #2171, Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-GM130 antibody (1:4000, NBP2-53420, Novus Biologicals, CO, USA). The membrane was reacted with the proper secondary antibodies for 60 min at room temperature. Chemiluminescence was developed using ECL Prime (GE Healthcare, Arlington Heights, IL, USA) and

detected by a CCD camera (Las-3000, Fuji photo, Tokyo, Japan).

3.2.4. Small RNA Sequencing

The total RNA of M-EVs was extracted using Total Exosome RNA & Protein Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA concentrations were measured using a Qubit™ High Sensitivity RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

A QIAseq miRNA Library Kit (Qiagen, Hilden, Germany) and QIAseq miRNA NGS Index TF (Qiagen, Hilden, Germany) were used to prepare the small RNA libraries. The detailed protocols are briefly described below. First, 10 ng of total RNA from the M-EVs were amplified for 17 cycles by PCR. Then, small RNA library concentrations were determined using a Qubit™ dsDNA Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). After that, the same concentrations of small RNA library were sequenced by 100 bp single-end using an Ion Torrent Ion GeneStudio S5 platform (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing data were processed using the GeneGlobe Data Analysis Center (Qiagen, Hilden, Germany). Then, the sequencing data were mapped to miRNA and piRNA using miRBase. For different RNA transcripts analysis, small RNA species lower than 9 reads were excluded.

3.2.5. Real-time PCR for miRNA Measurement

Taqman Advanced miRNA Assays were used to measure cel-miR-39-3p, miR-206-3p, miR-146a-5p, miR146b-5p, miR-486a-5p, miR-5131, and miR-6909-3p (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 8 ng of total RNA with 5'-phosphorylated oligonucleotide (cel-miR-39, Sequence: 5'-UCACCGGGUGUAAAUCAGCUUG-3') were reverse-transcribed using TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed using TaqMan Fast Advanced Master Mix with Fast 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR condition was below; denaturing at 95°C for 20 s followed by 40 cycles of denaturing at 95°C for 3 s, and annealing and elongation at 60°C for 30 s. All data were normalized to cel-miR-39-3p as spike-in control and calculated using ΔΔCt method.

3.2.6. Real-time PCR for mRNA Measurement

The protocol of real-time PCR for mRNA expression had be previously described [126], and a detailed protocol is described below. The total RNA of gastrocnemius was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The concentrations of total RNA were determined using NanoDrop 1000 (Thermo Fisher Scientific, Waltham,

MA, USA). The total RNA was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) with Fast 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR condition was below; denaturing at 95°C for 20 s followed by 40 cycles of denaturing at 95°C for 3 s, and annealing and elongation at 60°C for 30 s. All the data were normalized to 18s ribosomal RNA using the ΔΔCt method. The specific primer sequences are shown in Table 3.

Table 3. Primer sequences for real time-PCR.

	Forward	Reverse
18s	TTCTGGCCAACGGTCTAGACAAC	CCAGTGGTCTTGGTGTGCTGA
CD31	CCCTTCATTGACCTCAACTACAATGGT	GAGGGGCCATCCACAGTCTTCTG
CHOP	TATCTCATCCCCAGGAAACG	TATCTCATCCCCAGGAAACG
COX IV	TGGGAGTGTTGTGAAGAGTGA	GCAGTGAAGCCGATGAAGAAC
GADD34	CCTCTAAAAGCTCGGAAGGTACA	ATCTCGTGCAAACTGCTCCC
GLUT4	CCGCGGCCTCCTATGAGATACT	AGGCACCCCGAAGATGAGT
IL-1β	GGGCCTCAAAGGAAAGAATC	TTGCTTGGGATCCACACTCT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IRS1	ATCTTCCTTTGGCGCAGCTA	CAGCACGAAAAAGCGCTTA
Myh2	GAGCAAAGATGCAGGGAAAG	TAAGGGTTGACGGTGACACA
Myh4	GGGGCTGTACCAGAAATCCG	CCTGAAGAGAGCTGACACGG
Myh7	AGATGAATGCCGAGCTCACT	CTCATCCAAACCAGCCATCT
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Tfam	CCAAAAAGACCTCGTTCAGC	CTTCAGCCATCTGCTCTTCC
TNF-α	CCTCCCTCTCATCAGTTCTA	ACTTGGTGGTTTGCTACGAC
VEGF-A	GCACATAGGAGAGATGAGCTTCC	CTCCGCTCTGAACAAGGCT
XBP1 spliced	TGAGAACCAGGAGTTAAGAACACGC	CCTGCACCTGCTGCGGAC
XBP1 total	GAAGAAGAGAACCACAAACTCCAGC	ATCCAGCGTGTCCATTCCCA

CHOP, C/EBP homologous protein; COX IV, cytochrome c oxidase subunit IV; GADD34, growth arrest and DNA damage-inducible protein 34; GLUT4, glucose transporter type 4; IL, interleukin; IRS1, insulin receptor substrate 1; PGC-1α, peroxisome proliferator-activated receptor coactivator-1α; Tfam, mitochondrial transcription factor A; TNF,

tumor necrosis factor; VEGF-A, vascular endothelial growth factor A; XBP1, X-box binding protein 1.

3.2.7. Statistical Analysis

The data are presented as mean ± SE. The data were analyzed using a two-way ANOVA and then Bonferroni post hoc test. The statistical significance was defined as $p < 0.05$. The differently expressed small RNAs were analyzed using multiple t-test with an adjusted p-value (false discovery rate; FDR) < 0.1. Principal component analysis (PCA) was calculated from the RNA-seq data using singular value decomposition. Unit variance scaling was applied to the data. The scores plot was calculated and visualized using ClustVis (https://biit.cs.ut.ee/clustvis/) [127]. Statistical analysis was performed using SPSS V25.0 (IBM Japan, Ltd., Tokyo, Japan) or Prism (v.9.2.0, GraphPad, San Diego, CA, USA).

3.3. Results

3.3.1. The Effects of Voluntary Exercise Training and HFD Feeding on Body Weight and Muscle Weight

The mice were fed with normal diet ND or HFD and had free access to running wheels for 6 weeks. The mean voluntary running distance per day was not different between the ND feeding (10.0 ± 0.4 km, mean ± SE) and the HFD feeding (10.5 ± 0.5 km,

p = 0.518). The HFD feeding increased body weight, and the voluntary exercise training inhibited the increase of body weight by HFD feeding (Figure 6A). The gastrocnemius weight did not change by the voluntary exercise training but showed an increasing trend after the HFD feeding in sedentary mice (Figure 6B). The gastrocnemius weight per body weight showed an increasing trend after the voluntary exercise training and decreased after the HFD feeding in sedentary mice (Figure 6C).

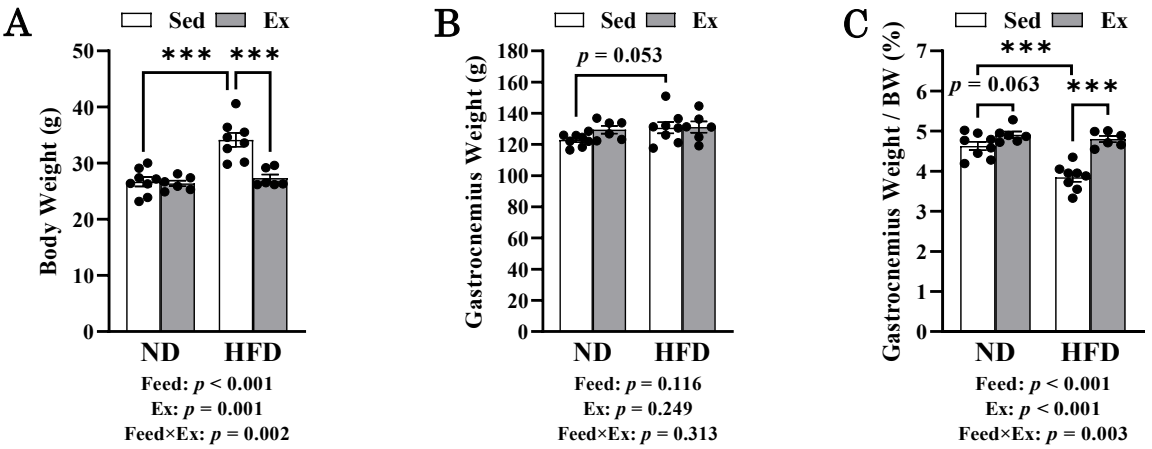


Figure 6. (A) Body weight, (B) gastrocnemius weight, and (C) gastrocnemius weight per body weight (n = 6-8). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [*** p < 0.001].

3.2.2. The Voluntary Exercise Training and the HFD Feeding does not Change the miRNA Profile in M-EVs

After the 6 weeks of voluntary exercise training and the HFD feeding, the gastrocnemius was incubated in serum-free DMEM for 24 h, and EVs were isolated from

the cultured medium. An EV marker alix could be detected in the M-EVs (Figure 7A). Also, a golgi-associated protein GM130, an intracellular protein, was detected (Figure 7A). Then, the present study carried out the small RNA-sequences and could detect 383 miRNAs and 64 piRNAs in M-EVs. The present study summed up the number of detected leads from all groups and observed that muscle-specific miRNAs such as miR-1a-3p, miR-133a-3p, and miR-206-3p were abundant in the M-EVs (Figure 7B).

The present study next investigated the effects of voluntary exercise training and HFD feeding on the small RNA profile in M-EVs. PCA showed greater overlap between all groups in PC1 (26.8%) and PC2 (18%) (Figure 7C). The present study next investigated differently expressed small RNAs in the M-EVs. However, the small RNAs did not significantly change between ND + Sed and ND + Ex, ND + Ex and HFD + Ex, and ND + Sed and HFD + Sed (data not shown). The present study picked up some miRNAs and measured them using real-time PCR. The voluntary exercise training and the HFD feeding did not change miR-206-3p, miR-146a-5p, and miR-486a-5p expression in the M-EVs (Figure 7D). The voluntary exercise training increased miR-146b-5p s expression in the M-EVs from HFD-fed mice (Figure 7D). MiR-5131 and miR-6909-3p expressions in the M-EVs were undetectable (data not shown). These results indicate that the effects of voluntary exercise training and HFD feeding on the miRNA profile in

M-EVs were negligible.

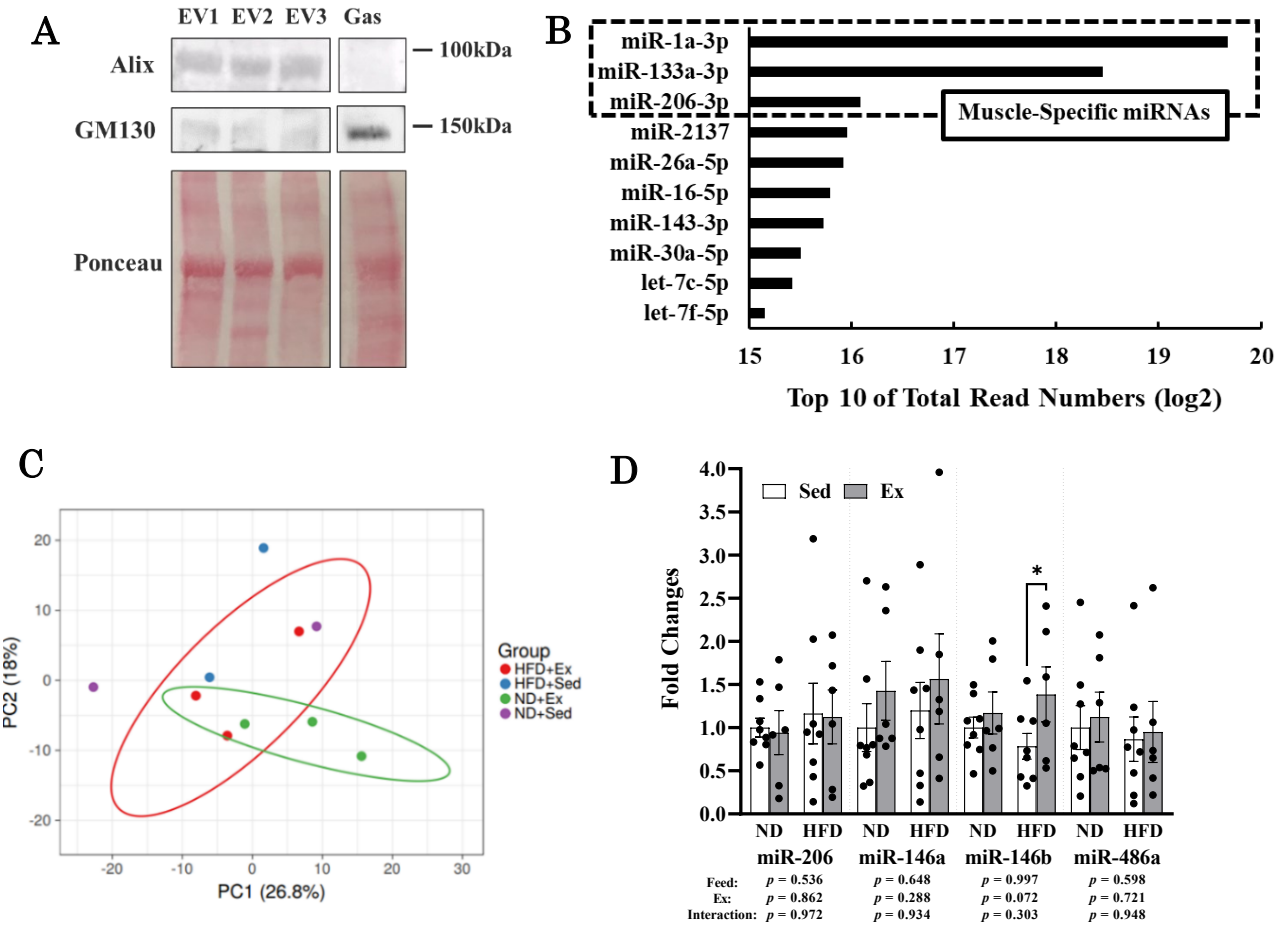


Figure 7. (A) Alix and GM130 protein expression in the M-EVs and gastrocnemius (n = 3). (B) Top 10 of total read numbers of miRNAs in the all groups of M-EVs evaluated by small RNA-seq. (C) Principal component analysis (PCA) plot about the small RNA-seq. Prediction ellipses show probability 0.95 from the same group (n = 2-3). (D) Determination of miRNA expression in M-EVs by real-time PCR (n = 6-8). Data are shown as mean ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc test [* $p < 0.05$].

3.2.3. Molecular Adaptations of the Voluntary Exercise Training and the HFD Feeding

Exercise training induces mitochondrial biogenesis, glucose metabolism enhancement, and angiogenesis in muscle [2]. However, the voluntary exercise training did not induce gene expression related to mitochondrial biogenesis (peroxisome

proliferator-activated receptor-gamma coactivator 1α, PGC-1α; mitochondrial transcription factor A, Tfam; cytochrome c oxidase subunit IV, COX IV), glucose metabolism (GLUT4; insulin receptor substrate 1, IRS1), and angiogenesis (CD31; vascular endothelial growth factor A, VEGF-A) (Figure 3A). Furthermore, voluntary exercise training also changes muscle fiber to oxidative fiber type [128]. However, in the present study, the voluntary exercise training did not change muscle fiber type-related gene expression (myh7, myosin heavy chain (MHC) I; myh2, MHC IIa; myh4, MHC IIb) (Figure 8B). HFD feeding and obesity induce muscle inflammation and endoplasmic reticulum (ER) stress [129–131]. However, in the present study, the HFD feeding did not change inflammatory cytokine gene expression (IL-1ß, IL-6, and TNF-α) (Figure 8C). Furthermore, the HFD feeding inhibited ER stress-related gene expression (total X-box binding protein 1, XBP1t; spliced XBP1, XBP1s; growth arrest and DNA damage-inducible protein 34, GADD34) (Figure 8D).

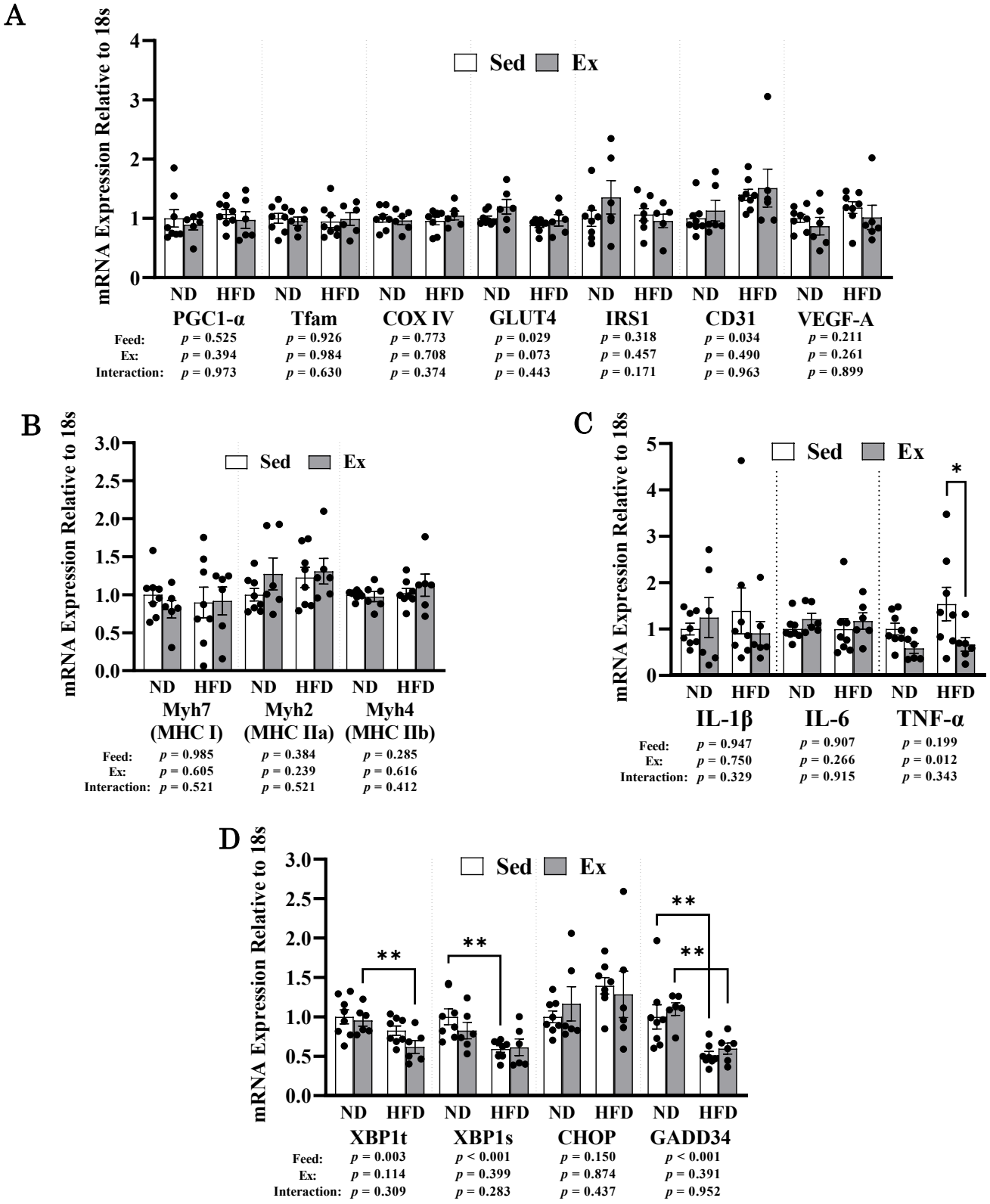


Figure 8. Gene expression of (A) exercise adaptation, (B) fiber type, (C) inflammation, (D) endoplasmic reticulum (ER) stress (n = 6-8). Data are shown as mean ± SE. Data

were analyzed using two-way ANOVA followed by Bonferroni post hoc test [* $p < 0.05$, ** $p < 0.01$].

3.3. Discussion

The present study used ex vivo explant muscle culture model to isolate M-EVs and could detect EV marker alix as previously reported [116,117,119,122–125]. However, M-EV isolation risks cellular component contamination using the ex vivo explant muscle culture model [116]. The present study also detected an intracellular golgi-associated protein GM130 in the M-EVs. Therefore, the data on the miRNA profile in M-EVs may be influenced by cellular component contamination. However, the other studies did not evaluate cellular component contamination [117,119,122–124]. Estrada et al. and the present study have shown that M-EV isolation using precipitation with volume-excluding polymers (e.g., Exoquick-TC by System Biosciences; Total Exosome Isolation Reagent from Cell Culture Media by Thermo Fisher Scientific) contaminates cellular components, but not using size-exclusion chromatography [116]. However, Mytidou et al. have reported that M-EV isolation using Exoquick-TC did not contaminate cellular components [125]. To our knowledge, the risk of cellular component contamination using other M-EV isolation methods (e.g., ultracentrifugation) is uninvestigated. The purity of M-EVs may depend on isolation methods and cultural conditions. Further study is

necessary to establish an isolation method of pure M-EVs.

The present study found that the 6 weeks of voluntary exercise training did not change the miRNA profile in M-EVs. This voluntary exercise period did not increase muscle weight but increased muscle weight per body weight. This voluntary exercise period also did not induce gene expression related to mitochondrial biogenesis, glucose metabolism, and angiogenesis. Therefore, this voluntary exercise period may not be long enough to increase muscle weight and induce molecular adaptations, which may not change the miRNA profile in M-EVs. The present study also showed that the 6 weeks of HFD feeding did not change the miRNA profile in M-EVs. One reason for this result may be an intervention period of the HFD feeding. HFD feeding induces muscle inflammation and ER stress, which inducing insulin resistance [129–131]. However, the 6 weeks of HFD feeding did not induce ER stress and inflammation and did not decrease glucose metabolism-related genes (GLUT4 and IRS1). Because these stresses were not induced, the miRNA profile in M-EVs may not change.

The present study showed that muscle-specific miRNAs such as miR-1, miR-133, and miR-206 were relatively rich in the M-EVs, consistent with the previous studies [117,118,122]. The reason for this result is that muscle-specific miRNAs are rich in muscle tissue [135] because Jalabert et al. has reported that the amount of miRNAs in

M-EVs is positively correlated with that in muscle tissue [123]. Therefore, the change of muscle miRNA may be a major regulator of miRNA in M-EVs. However, some miRNAs are specific to M-EVs [123]. Other factors may also regulate miRNA in M-EVs independent of the muscle miRNA levels. The present study obtained M-EVs from gastrocnemius. Gastrocnemius has both oxidative and glycolytic fiber. However, the characteristics of EVs are different from oxidative and glycolytic fiber [117,118,123]. Therefore, miRNA profile in M-EVs is different from oxidative and glycolytic fiber and may differ from our results obtained from gastrocnemius.

3.4. Conclusions

In the present study, 6 weeks of voluntary exercise training and HFD feeding do not change the miRNA profile in M-EVs, which is consistent with no changes in gene expression related to mitochondrial biogenesis, glucose metabolism, angiogenesis, and inflammation in muscle. The reason for this result may be shorter exercise training and HFD feeding intervention periods. Because the present study did not investigate these intervention periods, the present study do not conclude whether exercise training or HFD feeding miRNA profiles in M-EVs.

Chapter 4. The Impact of Acute Exercise on Amphiregulin and HB-EGF Expression

4.1. Introduction

Regular exercise has health-promoting effects and prevents various diseases. Although the mechanisms of these exercise effects are not completely understood, it has been revealed that muscle-derived humoral factors called myokine are secreted by exercise and regulate systemic health [136]. Recently, other than myokine, it has been revealed that exercise also secretes liver and adipose tissue-derived humoral factors, called hepatokine and adipokine, respectively [7,8]. Narrowly defined myokines are myocyte-derived humoral factors. However, exercise also induces IL-6, IL-1β, and IL-13 secretion from muscle leukocytes [12,30,51], which has an important role in muscle metabolic conditions [30,51]. Because the organs and cells other than myocyte and muscle secrete humoral factors by exercise, exercise-induced humoral factors were reorganized as exerkine [9]. Although many exerkines have been identified, the roles of exerkine are fully unrevealed.

Exercise training induces muscle growth via increased ribosomal function, satellite cells (SCs) activation, and mechanotransduction [137]. Myokine also regulates

muscle growth, and its representative myokines are IL-4, IL-6, IL-15, irisin, and myostatin [136–138]. In particular, acute exercise increases muscle and circulating IL-4 [52], IL-6 [136], IL-15 [139], and irisin [136,138]. Recently, epidermal growth factor (EGF) family proteins such as Areg and HB-EGF have been identified as muscle growth regulators [140–142]. Areg is secreted by regulatory T cells (Tregs) and promotes satellite cell differentiation and muscle regeneration [140]. HB-EGF is secreted by C2C12 myotube during differentiation and acts as a survival factor [141,142]. EGF family proteins, including Areg and HB-EGF, are ligands of EGF receptor (EGFR), which is expressed on SCs [137,140]. Therefore, Areg and HB-EGF may contribute to muscle growth via SCs activation. Furthermore, acute resistance and endurance exercises activate SCs and promote muscle growth [41,137]. Therefore, exercise may induce Areg and HB-EGF secretion. Although the previous study reported that exercise induces muscle HB-EGF 3 h after exercise [143], it is not understood the effects of acute exercise on these EGF family proteins over time in each organ. The present study aimed to elucidate the response of a single bout of exercise to EGF family.

4.2. Materials and Methods

4.2.1. Meta-analysis of Changes in Gene Expression by Exercise in Human Muscle

The present study used MetaMEx (https://www.metamex.eu/, version 2.2101) for meta-analysis of changes in human muscle gene expression induced by exercise [41]. The MetaMEx was set up to use data from immediately to 6 h after exercise (Immediately, 1 h, 3 h, 4 h, 5 h, and 6 h) and 18 h to 96 h after exercise (18 h, 24 h, 48 h, 96 h) with all exercise type (concentric, eccentric, and mix).

4.2.2. Animals

The present study used male C57BL/6J mice (aged 8-11 weeks) provided by Takasugi Experimental Animals Supply (Kasukabe, Japan). They were bred under a 12-h light/dark cycle. Food (MF, oriental yeast, Tokyo, Japan) and water were freely available to all the mice. This study was authorized by the Academic Research Ethical Review Committee of Waseda University (2021-A115).

4.2.3. Treadmill Running

The following exercise protocol was performed as previously described with minor modification [126], and a detailed protocol is described below. Four or five days

prior to the exhaustive exercise, all mice, including the sedentary group, were accustomed to treadmill running at 15 m/min and 7% grade for 10-15 min. After accustomed running, the mice in the exercise groups were made to run on a treadmill at 24 m/min and 7% gradient until exhaustion. After exhaustive exercise, the present study collected abdominal aortic blood samples containing heparin under isoflurane inhalation anesthesia (Abbott, Tokyo, Japan). The gastrocnemius, kidney, liver, and epididymal adipose tissues were collected after cervical dislocation and were then frozen in liquid nitrogen. Blood samples were centrifuged at 1600 g for 10 min at 4°C to extract plasma samples. All samples were maintained at −80°C.

4.2.4. Real-time PCR

The present study performed real-time PCR for mRNA expression as previously described [126], and a detailed protocol is described below. The detailed protocols are briefly described below. The total RNA of gastrocnemius was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The concentrations of total RNA were determined using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was

performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) with Fast 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR condition was below; denaturing at 95°C for 20 s followed by 40 cycles of denaturing at 95°C for 3 s, and annealing and elongation at 60°C for 30 s. All the data were normalized to 18s ribosomal RNA using the ΔΔCt method. The specific primer sequences are shown in Table 4.

Table 4. Primer sequences for real time-PCR.

	Forward	Reverse
18s	TTCTGGCCAACGGTCTAGACAAC	CCAGTGGTCTTGGTGTGCTGA
Areg	GGGGACTACGACTACTCAGAG	TCTTGGGCTTAATCACCTGTTC
BTC	AATTCTCCACTGTGTGGTAGCA	GGTTTTCACTTTCTGTCTAGGGG
HB-EGF	TCCGTCTGTCTTCTTGTCATCGT	TAGCCACGCCCAACTTCACT
TGF-α	CACTCTGGGTACGTGGGTG	CACAGGTGATAATGAGGACAGC

Areg, amphiregulin; BTC, betacellulin; HB-EGF, heparin-binding epidermal growth factor-like growth factor; TGF-α, transforming growth factor-α.

4.2.5. ELISA

The present study performed ELISA as previously described [126], and a detailed protocol is described below. To extract tissue proteins, the tissue was homogenized in tissue protein extraction reagent (T-PER; Thermo Fisher Scientific, Waltham, MA, USA) with a protease inhibitor (complete mini protease inhibitor cocktail tablet; Roche Diagnostics, Mannheim, Germany), centrifuged at 10,000 g for 15 min at 4°C, and obtained the protein containing supernatant. Then, the protein concentrations

in the supernatant were measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Areg and HB-EGF concentrations per total proteins were measured using Duoset ELISA kit (R&D Systems, Minneapolis, MN, USA). The absorbance was detected using Spectra Max iD5 (Molecular Devices Inc., San Jose, CA, USA).

4.2.6. Statistical Analysis

Data are expressed as mean ± SE. One-way ANOVA followed by Dunnett post hoc test was used for analyzing data. Statistical significance was defined as $p < 0.05$. Statistical analysis was performed using Prism (v.9.2.0, GraphPad, San Diego, CA, USA).

4.3. Results

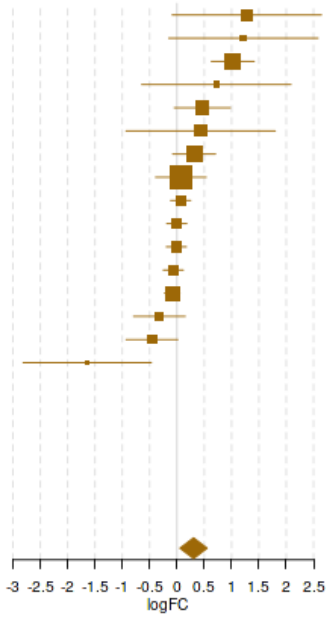
4.3.1. Acute Exercise Induces Muscle Areg and HB-EGF Gene Expression in Human

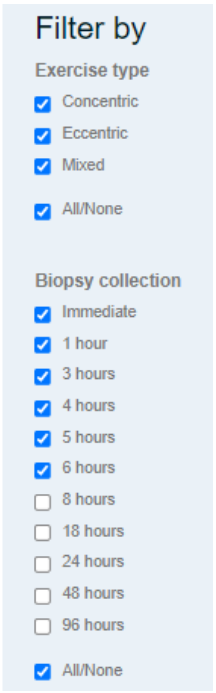
At first, the present study investigated the response of EGF family gene expression on acute exercise in human muscle using MetaMEx (https://www.metamex.eu/, version 2.2101), which is microarray and mRNA-Sequence based meta-analysis [41]. To investigate early exercise response of EGF family gene, the present study performed meta-analysis using the data from immediately to 6 h after

exercise. Meta-analysis showed that Areg and HB-EGF expression is increased 1.23-fold and 1.20-fold after aerobic exercise, respectively (Figure 9A,B). Furthermore, resistance exercise increases greater Areg and HB-EGF expression 2.69-fold and 3.71-fold, respectively compared to aerobic (Figure 9A,B). However, meta-analysis showed that transforming growth factor-α (TGF-α) and betacellulin (BTC) gene expression does not significantly change after aerobic and resistance exercise (data not shown). The present study next investigated exercise response of EGF family gene at recovery phase using the data from 18 h to 96 h after exercise. Meta-analysis showed that HB-EGF gene expression is increased 1.55-fold after resistance exercise in recovery phase (Figure 9C), but not significantly after aerobic exercise in recovery phase (Figure 9C). Areg, TGF-α, and BTC gene expression did not change after aerobic and resistance exercises in recovery phase (data not shown). The results of meta-analysis suggest that both aerobic and resistance exercise increases Areg and HB-EGF gene expression in human muscle.

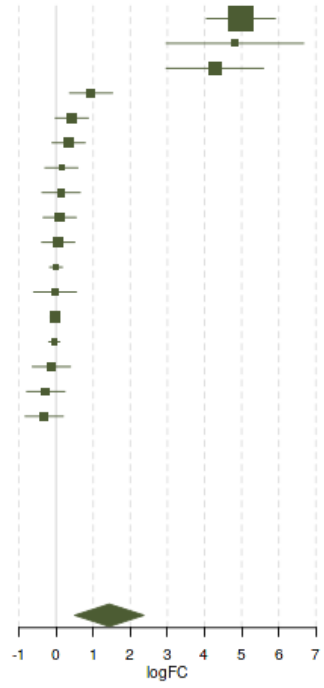
A

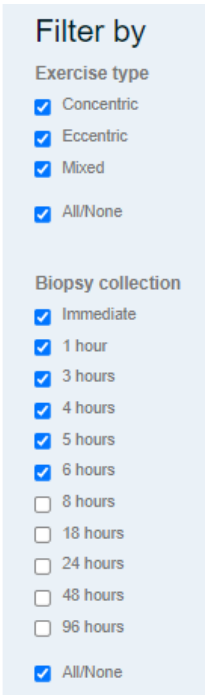
AREG in acute aerobic exercise studies	logFC	FDR	n
GSE71972_MIX_VAL_M_YNG_ACT_LEA_HLY_H03	1.28	4.0e-01	8
GSE71972_MIX_VAL_F_YNG_ACT_LEA_HLY_H03	1.22	4.1e-01	4
GSE33603_MIX_VAL_M_YNG_ACT_LEA_HLY_H03	1.02	9.4e-05	11
GSE71972_MIX_VAL_F_YNG_ACT_LEA_HLY_H00	0.72	1.0e+00	4
GSE41769_MIX_VAL_M_MDL_SED_OWE_HLY_H00	0.46	7.8e-01	9
GSE71972_MIX_VAL_M_YNG_ACT_LEA_HLY_H00	0.43	1.0e+00	8
GSE33603_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	0.31	7.1e-01	11
GSE43856_MIX_VAL_M_YNG_ATH_LEA_HLY_H03	0.07	9.8e-01	16
GSE59363_MIX_N.A_M_MDL_ACT_LEA_T2D_H00	0.07	9.8e-01	7
GSE59363_MIX_N.A_M_MDL_ACT_OWE_HLY_H00	0.00	1.0e+00	7
GSE59363_MIX_N.A_M_MDL_ACT_LEA_T2D_H03	-0.01	9.9e-01	7
GSE59363_MIX_N.A_M_MDL_ACT_OWE_HLY_H03	-0.07	1.0e+00	7
GSE87748_MIX_VAL_M_YNG_SED_LEA_HLY_H04	-0.09	8.9e-01	10
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H03	-0.32	7.0e-01	6
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H05	-0.46	5.8e-01	6
GSE86931_MIX_VAL_M_YNG_ATH_LEA_HLY_H04	-1.64	5.8e-01	2
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	NA	NA	
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	NA	NA	
GSE120862_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	NA	NA	
GSE120862_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	NA	NA	
GSE120862_MIX_VAL_M_YNG_SED_LEA_HLY_H01	NA	NA	
GSE120862_MIX_VAL_M_YNG_SED_LEA_HLY_H04	NA	NA	
GSE4247_MIX_VAL_M_YNG_SED_OWE_HLY_H03	NA	NA	
Meta-analysis score	**0.30**	**1.7e-02**	**123**





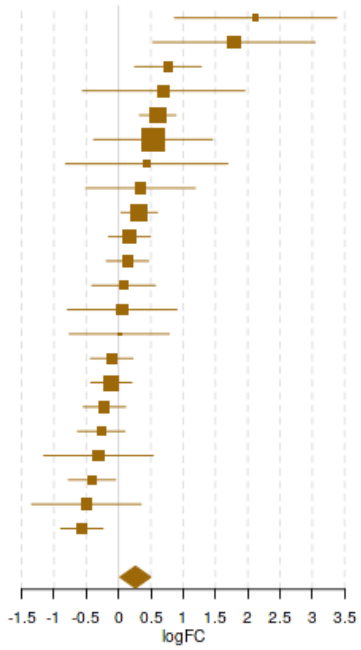
AREG in acute resistance exercise studies	logFC	FDR	n
GSE23697_ECC_VAL_M_YNG_SED_LEA_HLY_H03	4.98	5.4e-14	20
GSE23697_ECC_VAL_M_YNG_SED_OBE_HLY_H03	4.82	5.6e-04	5
GSE23697_ECC_VAL_M_YNG_SED_OWE_HLY_H03	4.28	1.8e-06	10
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H03	0.94	9.9e-02	6
GSE28422_MIX_VAL_F_YNG_SED_LEA_HLY_H04	0.42	7.2e-01	7
GSE28422_MIX_VAL_M_YNG_SED_LEA_HLY_H04	0.34	6.2e-01	8
GSE1832_MIX_VAL_M_MDL_SED_LEA_HLY_H06	0.14	1.0e+00	4
GSE28422_MIX_VAL_M_ELD_ACT_OWE_HLY_H04	0.14	1.0e+00	6
GSE28422_MIX_VAL_F_YNG_ACT_LEA_HLY_H04	0.10	8.4e-01	7
GSE28422_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	0.06	9.6e-01	8
GSE24235_MIX_BIB_M_YNG_SED_LEA_HLY_H04	0.00	1.0e+00	3
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H05	-0.03	9.9e-01	6
GSE19062_ECC_VAL_M_YNG_ACT_LEA_HLY_H03	-0.03	8.9e-01	8
GSE24235_MIX_BIB_F_YNG_SED_LEA_HLY_H04	-0.05	9.3e-01	4
GSE28422_MIX_VAL_M_ELD_SED_OWE_HLY_H04	-0.12	9.8e-01	6
GSE28422_MIX_VAL_F_ELD_SED_OWE_HLY_H04	-0.28	1.0e+00	6
GSE28422_MIX_VAL_F_ELD_ACT_OWE_HLY_H04	-0.32	1.0e+00	6
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	NA	NA	
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	NA	NA	
GSE4249_ECC_VAL_M_YNG_SED_LEA_HLY_H03	NA	NA	
GSE7286_CON_VAL_M_YNG_ACT_LEA_HLY_H03	NA	NA	
GSE7286_ECC_VAL_M_YNG_ACT_LEA_HLY_H03	NA	NA	
GSE7286_CON_VAL_M_YNG_ACT_LEA_HLY_H06	NA	NA	
GSE7286_ECC_VAL_M_YNG_ACT_LEA_HLY_H06	NA	NA	
Meta-analysis score	**1.43**	**2.5e-03**	**120**





B

HBEGF In acute aerobic exercise studies	logFC	FDR	n
GSE71972_MIX_VAL_F_YNG_ACT_LEA_HLY_H03	2.12	3.7e-02	4
GSE71972_MIX_VAL_M_YNG_ACT_LEA_HLY_H03	1.79	1.5e-01	8
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	0.77	1.8e-01	6
GSE71972_MIX_VAL_M_YNG_ACT_LEA_HLY_H00	0.70	1.0e+00	8
GSE33603_MIX_VAL_M_YNG_ACT_LEA_HLY_H03	0.61	1.3e-03	11
GSE43856_MIX_VAL_M_YNG_ATH_LEA_HLY_H03	0.54	8.4e-01	16
GSE71972_MIX_VAL_F_YNG_ACT_LEA_HLY_H00	0.44	1.0e+00	4
GSE120862_MIX_VAL_M_YNG_SED_LEA_HLY_H04	0.34	8.1e-01	7
GSE33603_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	0.32	3.4e-01	11
GSE41769_MIX_VAL_M_MDL_SED_OWE_HLY_H00	0.17	1.0e+00	9
GSE59363_MIX_N.A_M_MDL_ACT_OWE_HLY_H00	0.14	1.0e+00	7
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	0.08	9.9e-01	6
GSE120862_MIX_VAL_M_YNG_SED_LEA_HLY_H01	0.06	9.9e-01	7
GSE86931_MIX_VAL_M_YNG_ATH_LEA_HLY_H04	0.01	9.9e-01	2
GSE59363_MIX_N.A_M_MDL_ACT_LEA_T2D_H00	-0.11	9.8e-01	7
GSE87748_MIX_VAL_M_YNG_SED_LEA_HLY_H04	-0.11	8.9e-01	10
GSE59363_MIX_N.A_M_MDL_ACT_OWE_HLY_H03	-0.22	1.0e+00	7
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H03	-0.27	6.6e-01	6
GSE120862_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	-0.31	8.2e-01	7
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H05	-0.41	4.6e-01	6
GSE120862_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	-0.50	8.6e-01	7
GSE59363_MIX_N.A_M_MDL_ACT_LEA_T2D_H03	-0.57	2.8e-01	7
GSE4247_MIX_VAL_M_YNG_SED_OWE_HLY_H03	NA	NA	
Meta-analysis score	**0.26**	**3.3e-02**	**163**



Filter by

Exercise type

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- [x] Eccentric
- [x] Mixed
- [x] All/None

Biopsy collection

- [x] Immediate
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- [x] 3 hours
- [x] 4 hours
- [x] 5 hours
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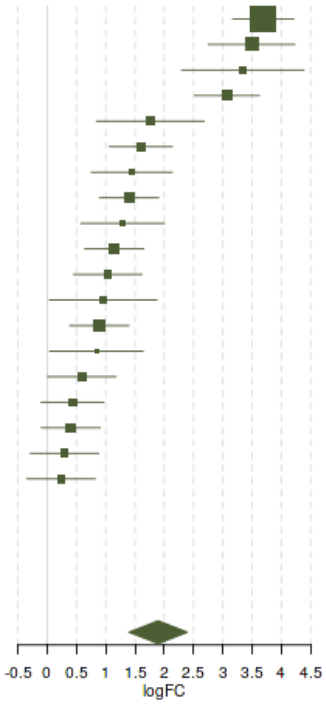
- [ ] 8 hours
- [ ] 18 hours
- [ ] 24 hours
- [ ] 48 hours
- [ ] 96 hours
- [x] All/None

HBEGF In acute resistance exercise studies	logFC	FDR	n
GSE23697_ECC_VAL_M_YNG_SED_LEA_HLY_H03	3.69	3.7e-19	20
GSE23697_ECC_VAL_M_YNG_SED_OWE_HLY_H03	3.49	4.1e-11	10
GSE23697_ECC_VAL_M_YNG_SED_OBE_HLY_H03	3.34	1.3e-05	5
GSE19062_ECC_VAL_M_YNG_ACT_LEA_HLY_H03	3.07	3.9e-08	8
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H05	1.76	2.9e-02	6
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	1.60	1.6e-04	6
GSE24235_MIX_BIB_F_YNG_SED_LEA_HLY_H04	1.45	6.8e-02	4
GSE28422_MIX_VAL_M_YNG_SED_LEA_HLY_H04	1.40	2.1e-04	8
GSE1832_MIX_VAL_M_MDL_SED_LEA_HLY_H06	1.29	1.0e+00	4
GSE28422_MIX_VAL_F_YNG_SED_LEA_HLY_H04	1.15	1.0e-02	7
GSE28422_MIX_VAL_M_ELD_ACT_OWE_HLY_H04	1.03	1.9e-01	6
GSE59088_MIX_VAL_M_YNG_SED_LEA_HLY_H03	0.96	4.1e-01	6
GSE28422_MIX_VAL_M_YNG_ACT_LEA_HLY_H04	0.89	6.2e-02	8
GSE24235_MIX_BIB_M_YNG_SED_LEA_HLY_H04	0.84	1.0e+00	3
GSE28422_MIX_VAL_M_ELD_SED_OWE_HLY_H04	0.59	7.2e-01	6
GSE107934_MIX_VAL_M_YNG_ACT_LEA_HLY_H01	0.43	9.3e-01	6
GSE28422_MIX_VAL_F_YNG_ACT_LEA_HLY_H04	0.40	4.2e-01	7
GSE28422_MIX_VAL_F_ELD_SED_OWE_HLY_H04	0.30	1.0e+00	6
GSE28422_MIX_VAL_F_ELD_ACT_OWE_HLY_H04	0.24	1.0e+00	6
GSE4249_ECC_VAL_M_YNG_SED_LEA_HLY_H03	NA	NA	
GSE7286_CON_VAL_M_YNG_ACT_LEA_HLY_H03	NA	NA	
GSE7286_ECC_VAL_M_YNG_ACT_LEA_HLY_H03	NA	NA	
GSE7286_CON_VAL_M_YNG_ACT_LEA_HLY_H06	NA	NA	
GSE7286_ECC_VAL_M_YNG_ACT_LEA_HLY_H06	NA	NA	
Meta-analysis score	**1.89**	**1.1e-13**	**132**



Filter by

Exercise type

- [x] Concentric
- [x] Eccentric
- [x] Mixed
- [x] All/None

Biopsy collection

- [x] Immediate
- [x] 1 hour
- [x] 3 hours
- [x] 4 hours
- [x] 5 hours
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- [ ] 8 hours
- [ ] 18 hours
- [ ] 24 hours
- [ ] 48 hours
- [ ] 96 hours
- [x] All/None

C

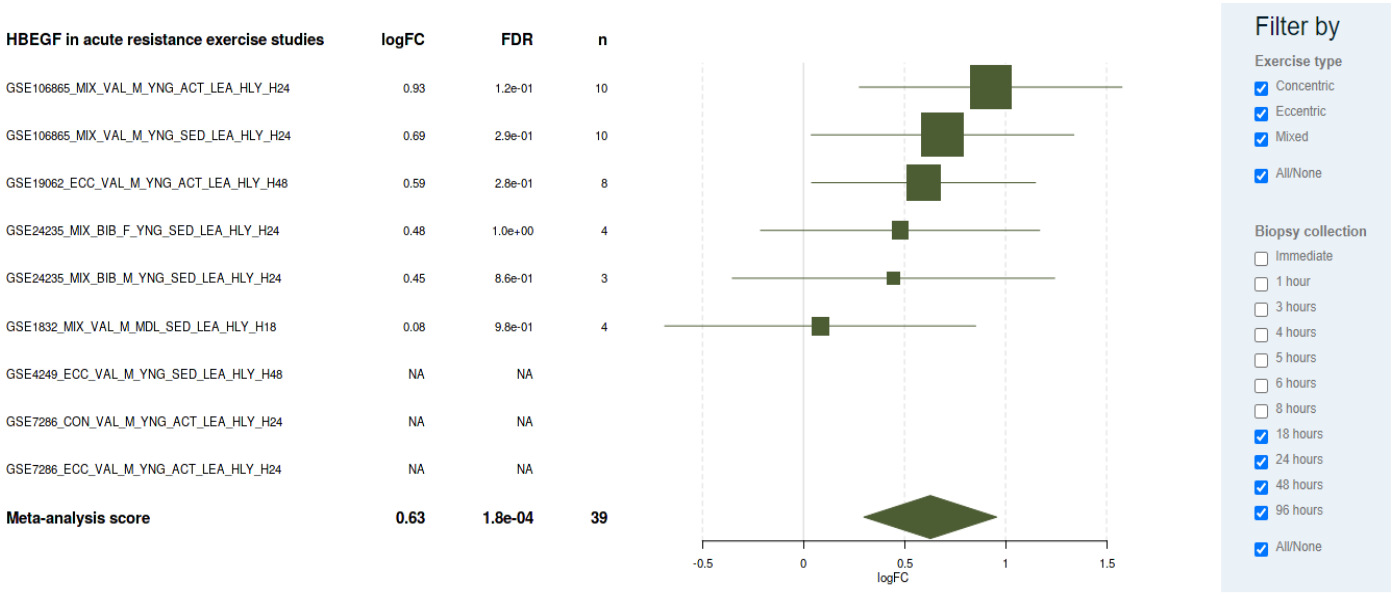

Figure 9. Meta-analysis of (A) Areg and (B) HB-EGF gene expression using the data from immediately to 6 h after aerobic and resistance exercise in human muscle analyzed by MetaMEx (https://www.metamex.eu/) [41]. (C) Meta-analysis of HB-EGF gene expression using the data from 18 h to 96 h after resistance exercise. logFC means log2 fold change. Areg, amphiregulin; HB-EGF, heparin-binding epidermal growth factor-like growth factor.

4.3.2. The Effects of Exhaustive Exercise on EGF Family Expression in Murine Muscle

The present study next investigated the effects of exhaustive treadmill running (time to exhaustion; 209.1 ± 10.1 min, mean ± SE) on murine muscle EGF family gene expression. Exhaustive exercise increases Areg and HB-EGF gene expression (Figure 10A). Other EGF family, BTC and TGF-α, gene expression decreased after exercise (Figure 10A). However, HB-EGF protein levels did not change after exercise (Figure 10B). Areg protein concentrations were undetectable (data not shown), and plasma Areg and HB-EGF concentrations also undetectable (data not shown).

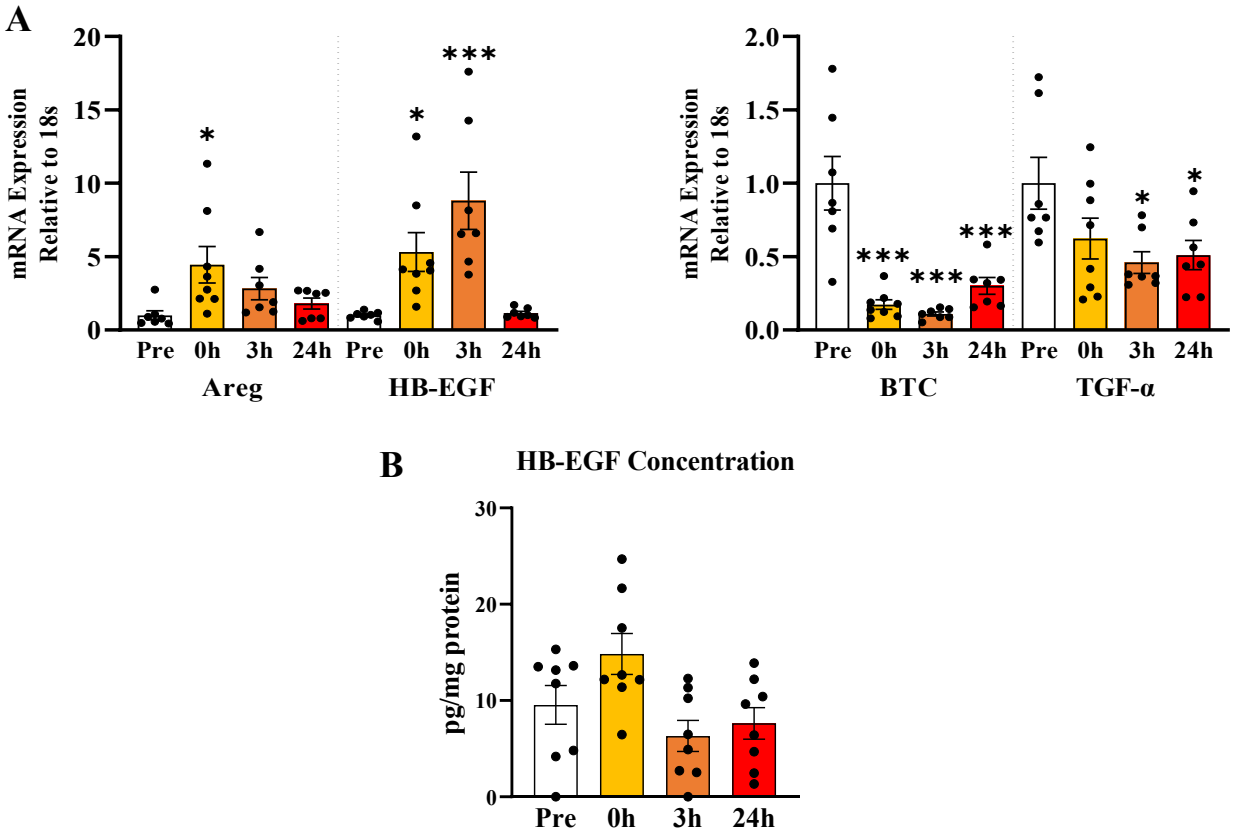

Figure 10. (A) Gene expression of EGF family in the gastrocnemius (n = 7-8). (B) HB-EGF concentration in the gastrocnemius (n = 7-8). Data are shown as mean ± SE. Data were analyzed by one-way ANOVA followed by Dunnett post hoc test compared to Pre [* $p < 0.05$, *** $p < 0.001$]. Areg, amphiregulin; BTC, betacellulin; HB-EGF, heparin-binding epidermal growth factor-like growth factor; TGF-α, transforming growth factor-α.

4.3.3. The Effects of Exhaustive Exercise on Areg and HB-EGF Protein Concentrations in Murine Liver, Kidney, and Adipose Tissue

The present study next investigated the impacts of exercise on Areg and HB-EGF protein concentrations in liver, kidney, and adipose tissue. Areg protein concentrations increased 24 h after exercise in liver and decreased 3 h and 24 h after exercise in adipose tissue (Figure 11A). Kidney HB-EGF protein concentrations decreased 24 h after exercise (Figure 11B).

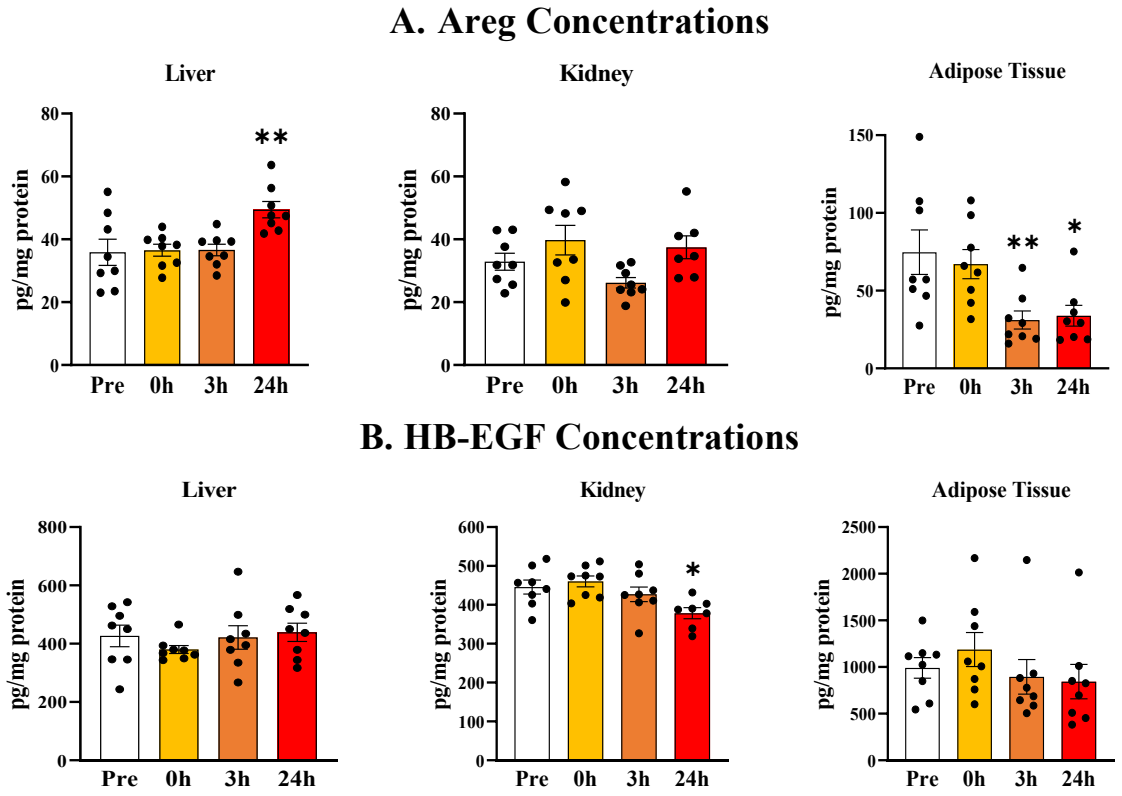

Figure 11. (A) Areg and (B) HB-EGF protein concentrations in liver, kidney, and adipose tissue (n = 7-8). Data are shown as mean ± SE. Data were analyzed by one-way ANOVA followed by Dunnett post hoc test compared to Pre [* $p < 0.05$, ** $p < 0.01$]. Areg, amphiregulin; HB-EGF, heparin-binding epidermal growth factor-like growth factor.

4.3.4. The Changes in the Blood Biochemical Parameters After Exhaustive Exercise

The present study next investigated the effects of exhaustive exercise on organ damage and metabolic state. Exhaustive exercise increased the plasma levels of muscle damage marker creatine kinase (CK), liver damage marker (aspartate aminotransferase; AST), and kidney damage markers (creatinine and blood uric nitrogen; BUN) (Table 5). Exhaustive exercise also increased the plasma levels of cellular damage marker lactate dehydrogenase (LDH) (Table 5). Furthermore, exhaustive exercise changed the plasma levels of several metabolic parameters such as free fatty acids (FFA), glucose, total cholesterol, and high-density lipoprotein (HDL) cholesterol (Table 5).

Table 5. The blood biochemical data.

	Pre	0h	3h	24h
CK (IU/L)	140.25 ± 38.65	3417.375 ± 1201.12**	678.375 ± 106.79	168.75 ± 29.28
AST (IU/L)	52.12 ± 8.18	220.87 ± 37.03	483.00 ± 128.90***	90.00 ± 13.02
ALT (IU/L)	22.50 ± 3.05	64.87 ± 9.19	205.50 ± 125.65	31.13 ± 3.05
BUN (mg/dL)	23.55 ± 0.70	47.63 ± 3.59***	35.55 ± 1.97*	20.03 ± 1.15
Creatinine (mg/dL)	0.15 ± 0.01	0.23 ± 0.02*	0.17 ± 0.01	0.12 ± 0.01
LDH (IU/L)	173.63 ± 18.28	861.00 ± 144.23	1270.88 ± 375.71**	242.63 ± 22.52
TG (mg/dL)	55.50 ± 4.09	59.63 ± 5.08	37.87 ± 5.75	60.37 ± 10.23
FFA (μg/dL)	1.76 ± 0.09	2.54 ± 0.10***	1.47 ± 0.17	1.59 ± 0.10
Glucose (mg/dL)	271.13 ± 10.70	140.25 ± 20.27***	214.88 ± 11.65*	276.75 ± 10.98
Total Cholesterol	82.88 ± 1.88	70.88 ± 2.94*	73.13 ± 3.40	97.88 ± 3.76**
HDL Cholesterol	67.50 ± 2.41	57.00 ± 3.31*	61.50 ± 3.35	77.63 ± 2.69
LDL Cholesterol	9.00 ± 0.98	7.88 ± 0.55	7.13 ± 1.13	11.25 ± 1.24

Data are shown as mean ± SE. Data were analyzed by one-way ANOVA followed by Dunnett post hoc test compared to Pre [* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$]. CK, creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; TG, triglyceride; FFA, free fatty acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

4.4. Discussion

In the present study, the present study found that acute exercise induces Areg and HB-EGF gene expression in human and murine muscles. Meta-analysis showed that resistance exercise induces greater gene expression than aerobic exercise. In particular, eccentric exercise is higher Areg and HB-EGF gene expression (GSE23697, GSE19062). Therefore, muscle damage may induce muscle Areg and HB-EGF gene expression. In the present study, the increase of Areg and HB-EGF gene expression coincides with the increase of plasma CK levels. However, there is no correlation between CK and Areg or

HB-EGF immediately after exercise (CK vs. Areg, r = 0.342, p = 0.408; CK vs. HB-EGF, r = -0.457, p = 0.255). These results suggest that muscle damage is not strongly associated with Areg and HB-EGF gene expression.

Although exercise induces Areg and HB-EGF gene expression in muscle, the source of Areg and HB-EGF is unrevealed. Because cardiotoxin-induced muscle injury induces the infiltration of Areg expressing Tregs [140], one candidate of Areg-secreting cells may be Tregs. However, these Tregs infiltration in muscle peaks in regeneration phase (4-30 days after cardiotoxin-induced muscle injury) but not early inflammatory phase (1 day after muscle injury) [140]. In the present study, muscle Areg gene expression increased only immediately after exercise. These results suggest that, at least, infiltrating Tregs are not Areg-secreting cells. Other candidate is M1 macrophages because M1 macrophages secrete Areg in vitro [144], and macrophages infiltrate into muscle after exercise [12]. Neutrophils are also a candidate as the Areg-secreting cells because exercise induces Areg gene expression in human neutrophils [145], and neutrophil infiltrates into muscle after exercise in mice [126]. Further study is necessary to identify Areg-secreting cells. In different from the change that Areg gene expression peaks immediately after exhaustive exercise, HB-EGF gene expression peaks 3 h after exercise in murine muscle. Meta-analysis also indicates HB-EGF gene expression

increased in recovery phase in human muscle, but not Areg gene expression. Therefore, HB-EGF-secreting cells may be different from Areg-secreting cells. HB-EGF is produced by various types of cells such as macrophages and myotubes [142,146]. Therefore, these cells may be the source of HB-EGF. However, to our knowledge, there is no study investigating the source of exercise-inducible HB-EGF.

Contrary to the change in gene expression, the present study showed that muscle Areg protein concentrations are undetectable, and muscle HB-EGF protein concentrations did not change after exercise in mice. Another possibility is that because these proteins were released into the circulation, these protein concentrations did not change in muscle. However, plasma concentrations of Areg and HB-EGF were also undetectable in the present study. Therefore, Areg and HB-EGF may not have physiological roles in muscle. On the other hand, Fukatsu et al. have reported that acute exercise increases muscle HB-EGF proteins detected by western blot [143]. These methodological differences may result in this discrepancy.

In the present study, exhaustive exercise increased liver Areg concentrations. A previous study has reported that Areg is induced by acute liver injury [147]. In the present study, exhaustive exercise increases plasma AST, which suggests that our exercise protocol induced liver damage. Therefore, exercise-induced liver damage may

be one factor for upregulating liver Areg. Areg has various roles in cell proliferation, apoptosis, migration, and tissue repair [148,149], and Areg is also an important factor for liver regeneration [150]. Considering these results, exercise-induced liver Areg induction may contribute to liver repair from exercise-induced liver damage. In the present study, exhaustive exercise decreased adipose tissue Areg concentrations and kidney HB-EGF concentrations. However, it is difficult to infer the cause and physiological significance. Some studies have reported that obesity upregulates adipose tissue Areg expression [151,152], and Areg may be related to lipolysis or lipogenesis.

Although plasma Areg and HB-EGF concentrations were undetectable in the present study, these proteins increased in liver or kidney after exercise. Based on Areg and HB-EGF being humoral factors, these proteins may act in a paracrine/autocrine manner. In contrast to liver, adipose tissue Areg levels decreased after exercise. Ishiuchi et al. have reported exercise-reducible myokine CXCL10 and CCL5 [153,154]. Considering these researches, adipose tissue Areg may also be an exercise-reducible exerkine.

EGFR is a main receptor for EGF family proteins [146]. When EGF family proteins bind to EGFR, EGFR is phosphorylated [146]. Then, signal transduction proceeds [146]. In the present study, exhaustive exercise modulated liver and adipose

tissue Areg and kidney HB-EGF. Therefore, exercise may modulate EGFR signal transduction.

4.4. Conclusions

The present study investigated the change of Areg and HB-EGF following acute exercise in each organ. Then, the present study showed acute exercise induces muscle Areg and HB-EGF gene expression but not protein levels. Moreover, acute exercise modulates Areg levels in liver and adipose tissue and HB-EGF level in kidney but not in circulation. Because Areg and HB-EGF are humoral factors and modulated by acute exercise, these proteins may be able to define as exerkine to act locally. Further studies are necessary to investigate the importance of Areg and HB-EGF as exerkines.

Chapter 5. Conclusion

5.1. New Findings of the Studies Related to the Doctoral Thesis

This doctoral thesis aimed to resolve the problem as described below; (2) The studies of exerkine receptors are insufficient, and (3) It is necessary to search for novel exerkine through the development of analytical technology. Then, this chapter summarized new findings of this thesis below.

The first study investigated the role of CCR2 on exercise-induced inflammation in mice. Acute exercise with blockade of CCR2 exacerbates the inflammatory gene and protein expressions in muscle, kidney, liver, and adipose tissue immediately after exercise. Moreover, acute exercise with blockade of CCR2 exacerbates the inflammatory gene expression and M1 macrophage infiltration in muscle 24 h after exercise. From these results, this study concluded that blockade of CCR2 using CCR2 antagonist exacerbates exercise-induced inflammation in mice. However, this study may observe the effects other than CCR2 blockade because CCR2 antagonist RS-504393 used in this study has an antagonizing activity against the α1-adrenergic receptor [110,111]. Further studies are necessary to investigate the role of CCR2 signaling using more specific methods (e.g., CCR2 KO mice).

The second study investigated the effects of exercise training and HFD feeding

on miRNA profiles in M-EVs. This study had mice 6 weeks of voluntary exercise training. Then, this study analyzed the miRNA profile in M-EVs obtained using ex vivo explant muscle culture model. However, the small RNA-seq indicated that 6 weeks of exercise training and HFD feeding did not change miRNA profile in M-EVs. Therefore, this study concluded that 6 weeks of voluntary exercise training and HFD feeding do not change the miRNA profile in M-EVs. Furthermore, this study could not identify exercise training-inducible miRNAs in M-EVs as exerkines. One reason for this result may be that exercise training intervention is short. Further studies are necessary to investigate the exercise training term inducing the change of miRNAs in M-EVs.

The third study investigated the effects of acute exercise on EGF family proteins in each organ. In this study, acute exercise induces Areg and HB-EGF gene expression in both human and murine muscle but not protein levels in murine muscle. Furthermore, acute exercise modulates Areg and HB-EGF levels in each organ but not in circulation. Because Areg and HB-EGF are humoral factors and modulated by acute exercise, these proteins may be able to define as exerkines to act locally. Further studies are necessary to investigate the role and importance of Areg and HB-EGF as exerkines.

The present study elucidated the physiological roles of CCR2 signaling and the importance of investigating the roles of exerkine receptor. Although there are some

studies about the physiological roles of exerkine receptors, such as IL-6R and IL-13R [26,51], their studies are insufficient. The present study also elucidated exercise response of novel exerkine candidates, EVs, HB-EGF, and Areg. In conclusion, this doctoral thesis showed there is the potential for exploring further novel exerkines and investigating the role of exerkine receptors in addition to exerkines is also important.

5.2. Further Perspective

Exercise is effective not only in preventing disease and health promotion but also in improving several diseases, including type 2 diabetes [1]. Therefore, the development of new drugs can be expected by investigating the efficacy of exerkine on these diseases. In addition, investigation of these gene polymorphisms will be useful for elucidating individual differences in the exercise effects and for providing exercise prescriptions tailored to individual characteristics.

However, one problem must be resolved for exerkine to be useful for human health. It is whether the roles of exerkines are saved across species because exerkine research frequently uses rodents for the analysis of exerkines functions (e.g., genetically modified mice). Electrical pulse stimulation-induced muscle cells contraction model mimics exercise and is effective in searching for novel exerkines, particularly myokines.

However, this model frequently uses in murine myotubes (differentiated C2C12 cells or primary myoblasts) because human myotubes (differentiated primary myoblasts) are poorer contractive activity than that of murine myotubes [92]. Therefore, it is necessary to investigate that identified exerkines in other species are also important in humans.

In recent exerkine studies, exerkines released into circulation in an endocrine manner have been well focused. Because humoral factors also act in a paracrine manner, there may be exerkines acting in a paracrine manner. However, the investigation of these exerkines is not enough. Further studies are necessary to identify the novel exerkines acting in a paracrine manner.

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