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Macrophage Depletion Attenuates Acute Renal Damage after Exhaustive Exercise in Mice

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ABSTRACT

Exhaustive exercise is known to induce acute renal damage. However, the precise mechanisms remain unclear. We investigated the effects of macrophage depletion on exhaustive exercise-induced acute renal damage. Male C57BL/6 J mice were divided into four groups: sedentary with control liposome (n = 8), sedentary with clodronate liposome (n = 8), exhaustive exercise with control liposome (n = 8), and exhaustive exercise with clodronate liposome (n = 8). Mice were treated with clodronate liposomes or control liposomes intraperitoneally for 48 h before undergoing exhaustive exercise. Renal function and renal histology were tested at 24 h. The expression levels of kidney injury molecule (KIM)-1 and inflammatory cytokines in kidney tissues were measured by quantitative RT-PCR, and KIM-1 concentration was semi-quantified by immunostaining. As a result, exhaustive exercise increased macrophage infiltration into the kidney. However, clodronate reduced it. Although exhaustive exercise resulted in an increase in KIM-1 mRNA expression levels and concentration, injection of clodronate liposome reduced it. In addition, TUNEL positive apoptotic cells were increased after exercise, but significantly reduced by clodronate. Clodronate liposome treatment also decreased the mRNA expression levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the kidney after exhaustive exercise. These results suggest that macrophages play a critical role in increasing renal damage by regulating inflammation.

Introduction

Moderate exercise is effective for maintaining or promoting health [1], whereas intense prolonged exercise is related to health problems such as muscle damage and acute renal damage [2, 3]. It was shown that gross hematuria and microscopic hematuria were observed in 20% of post-race marathon runners [4]. In addition, we reported the effects of a duathlon race in humans in whom acute renal damage was demonstrated by increased serum creatinine levels and urinary protein, and tubular epithelial cells were also detected in urinary sediments [5].

In order to elucidate the precise mechanisms of renal damage after intense exercise, appropriate samples are required. However,

there is limited availability in humans owing to ethical considerations. In animal models, running on a treadmill or swimming are employed in mice and rats. Lin et al. reported acute renal damage occurred 24 hours after exhaustive exercise in rats. They showed that renal dysfunction was demonstrated by elevated levels of blood urea nitrogen (BUN) and creatinine in plasma, and histological damage was demonstrated by enlarged glomeruli, collapsed tubular epithelial cells, loss of brush border membranes in proximal epithelial cells, dilatation of tubules, and intratubular cast formation [6]. Wu et al. also reported acute renal damage occurred after exhaustive swimming in rats. They showed increased apoptosis was demonstrated by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining; inflammatory responses were demonstrated by increased tumor necrosis factor (TNF)-α and nuclear factor kappa B (NF-κB) activation [7]. Thus, it is assumed that inflammatory responses are responsible for the acute renal damage observed after intensive exercise. Interestingly, several studies reported that substances with anti-inflammatory properties were effective in suppressing acute renal damage following exhaustive exercise [6, 7], suggesting that inflammation may be of central importance in the induction of acute renal damage.

During intensive exercise, it is known that blood flow in the muscle increases, whereas that of the kidney or intestines decreases. Thus, intensive exercise might induce acute renal damage that resembles a hypoxia/reperfusion-induced acute kidney injury [6]. On the other hand, some systemic changes should be considered including dehydration and systemic inflammation due to muscle and intestinal damage; however, precise mechanisms of renal damage after intense exercise have not been fully elucidated.

Macrophages are recruited into the inflamed tissues and contribute to tissue damage and inflammation via secretion of inflammatory cytokines [8]. When acute kidney injury is induced by the hypoxia/reperfusion model, macrophages are recruited into the kidney and inflammatory cytokines and chemokines are increased [9]. On the other hand, macrophage depletion by clodronate improved acute kidney injury induced by hypoxia/reperfusion [10, 11]. Jo et al. also reported that macrophage depletion by clodronate improved acute kidney injury induced by hypoxia/reperfusion accompanied with reduced apoptosis and production of inflammatory cytokines and chemokines [12]. Therefore, the exhaustive exercise-induced infiltration of macrophages might be an important factor in the development of renal damage; however, it has not been demonstrated whether macrophages can promote renal damage following exhaustive exercise. Here, we hypothesized that macrophage depletion by peritoneal administration of clodronate liposomes may also ameliorate acute renal damage after exhaustive exercise, and assessed renal function, renal histology, inflammatory responses, and apoptosis 24 hours after exhaustive exercise.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from Kiwa Laboratory Animals (Wakayama, Japan) at 10 weeks of age and were housed in groups of four mice per cage in a controlled environment, under a light/dark cycle (lights on at 9:00 and off at 21:00). The experimental procedures complied with the Guiding Principles for the Care and Use of Animals in Waseda University and were approved by the Institutional Animal Care and Use Committee in the university (2013-A110). The mice were randomly assigned to four groups: sedentary with control liposome (n = 8), sedentary with clodronate (n = 8), exhaustive exercise with control liposome (n = 8), and exhaustive exercise with clodronate (n = 8). All the mice had free access to standard chow and water.

Macrophage depletion

To deplete macrophages, 150 µL of Clophosome-A – Clodronate Liposomes (Anionic) (Funakoshi, Tokyo, Japan) was administered intraperitoneally under anesthesia with 2% isoflurane inhalation at 0.8 L/min (Abbott Japan, Tokyo, Japan) using a gas anesthesia system for small laboratory animals (DS Pharma Biomedical, Osaka, Japan). Control animals were administered 150 µL of plain control liposomes for Clophosome-A (Funakoshi) in the same conditions.

Exercise protocol

Mice in the sedentary groups remained in resting conditions in the cage, whereas mice in the exercise groups were subjected to exhaustive exercise 48 h after the injection. One week before undergoing the exhaustive exercise, mice in all groups were familiarized with running on a motorized treadmill (Natsume, Tokyo, Japan). On the day of the experiment, the mice were forced via a shock grid to run on a treadmill with a 7% gradient and the speed set to 10 m/min for 15 min, followed by 15 m/min for 15 min, and 20 m/min for 15 min each, and finally at 24 m/min until exhaustion. Exhaustion was defined as the point when the mice refused to run despite being given the shock grid five times.

Blood and kidney sampling

Animals were sacrificed at 24 h after exhaustive exercise in all groups. Anesthesia was induced with 2% isoflurane inhalation at 0.8 L/min, and maintained with 1% at 0.8 L/min. Blood samples were obtained using heparin via the abdominal aorta, centrifuged at 2600 g for 10 min and plasma was stored at –80 °C until analysis. The kidneys were removed and the right kidneys were snap frozen by immersing the samples in liquid nitrogen and stored at –80 °C until analysis; the left kidneys were frozen in Tissue-tek Crymold (Sakura, Torrance, CA, USA) filled with OCT compound (Sakura) by immersing the samples in precooled isopentane at –80 °C.

Assessment of renal function

The plasma concentrations of BUN and creatinine were analyzed by Oriental Yeast Co., Ltd (Tokyo, Japan).

Histological analysis

Serial kidney sections 3 µm thick were used for staining and were analyzed by microscope (Biozero BZ-8100; Keyence, Osaka, Japan).

Hematoxylin and eosin staining

The kidney specimens were fixed in 10% paraformaldehyde before being embedded in paraffin. The specimens were sectioned at 3-µm, deparaffinized, and stained with hematoxylin and eosin for light microscopic analysis.

Immunohistochemistry

Immunohistochemistry was performed to examine the expression of F4/80, monocyte chemoattractant protein-1 (MCP-1), and kidney injury molecule (KIM)-1. The specimens were sectioned at 3 μm and deparaffinized and stained using ImmunoCruz rabbit ABC Staining System (Santa Cruz Biotechnology, Dallas, TX, USA). The primary anti-bodies used were rabbit anti-mouse F4/80 monoclonal antibody (M4150; Spring Bioscience, Pleasanton, CA, USA), rabbit anti-MCP-1 antibody (ab25124; Abcam, London, UK), and rabbit anti-KIM-1 antibody (ab47635; Abcam). F4/80 positive cells were counted on 4 random high power (200 ×) fields/slide using BZ-2 software (Keyence).

TUNEL assay

A TUNEL assay for the detection of apoptotic cells was performed with apoptosis in situ detection kit (Wako, Osaka, Japan) according to the manufacturer's protocol. The specimens were sectioned at 3 μm and deparaffinized and stained; then the images were visualized by BZ-8100 (Keyence). Each of 4 randomly selected images were recorded at 200 × magnification and analyzed by BZ-2 software (Keyence).

KIM-1 assay

The renal concentration of KIM-1 was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam). The assay procedures were performed according to the ELISA kit instructions.

Quantitative RT-PCR

Total RNA was extracted from the kidney using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Quantitative RT-PCR was performed with the Fast 7500 real-time PCR system (Applied Biosystems) using Fast SYBR Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 3 sec, and annealing at 60 °C for 15 sec. The 18 S ribosomal RNA was used as the housekeeping control, and all the data were normalized by the expression of 18 S ribosomal RNA. The data were expressed as the number of fold changes relative to the values of the sedentary with the control liposome group. The specific PCR primer pairs for each gene are shown in ▶ **Table 1**.

Statistical analyses

All data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using version 19.0 of the Statistical Package for Social Sciences software (IBM Corp., Armonk, NY, USA). To evaluate the statistical significance of the exhaustive exercise and macrophage depletion, the data were determined using two-way ANOVA. If significant interactions were observed, further comparisons were performed using the Tukey's HSD post hoc test. The level of significance was set at $P < 0.05$.

Results

Running time

The mean running time until the mice became exhausted was 161.0 ± 14.2 min in the control liposomes groups and 187.7 ± 13.8 min in the clodronate groups, which were not statistically different.

Macrophage Infiltration in the kidney

To identify the effect of clodronate treatment on exhaustive exercise-induced macrophage infiltration, we examined immunochemistry staining and mRNA expression of F4/80, which is a specific maker of macrophage, and MCP-1, which recruits monocytes and macrophages to the sites of inflammation. The number of F4/80 positive cells was significantly higher in exhaustive exercise group compared to sedentary group. However, the F4/80 positive cells were markedly decreased in the exhaustive exercise with clodronate liposome group (▶ **Fig. 1a, b**). Similarly, while exhaustive exercise increased the F4/80 mRNA in the kidney, injection of clodronate liposome reduced it (▶ **Fig. 1b**).

In addition, MCP-1 immunohistochemical staining showed that the expression of MCP-1 in the exercise with control liposomes group (▶ **Fig. 1a**) was increased primarily in the tubular epithelial cells compared with the sedentary with control liposomes group (▶ **Fig. 1a**). The expression of MCP-1 was low in both the exercise with clodronate group and sedentary with clodronate group (▶ **Fig. 1a**). The main effect of exercise on MCP-1 mRNA expression was observed (▶ **Fig. 1c**).

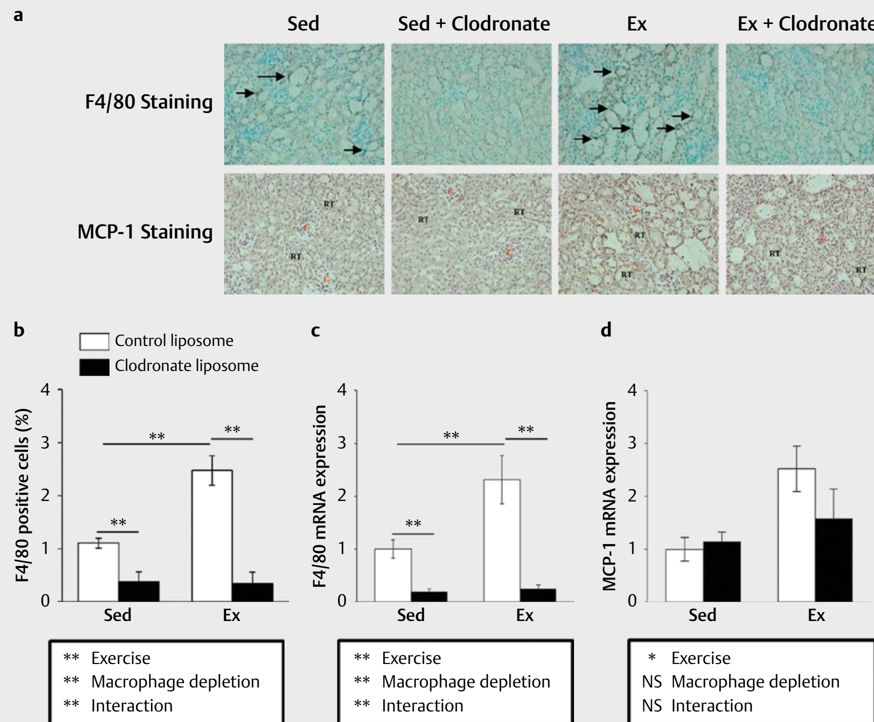
Renal function

The effects of exhaustive exercise and macrophage depletion on renal function were assessed with the plasma levels of BUN and creatinine 24 h after exercise. There was no significant difference in the levels of BUN and creatinine in each group (▶ **Table 2**).

▶ **Table 1** Primer sequences for RT-PCR analysis.

Gene	Forward	Reverse
18 s ribosomal RNA	CGGCTACCACATCCAAGGA	AGCTGGAATTACCGCGGC
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAG-TTTATCGTG
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
KIM-1	AAACCAGATTCCCACACG	GTCGTGGGTCTTCCTGTAGC
TNF-α	TCTTCTCATTCCTGCTTGTGG	GAGGCCATTTGGGAACTTCT
IL-6	AACGATGATGCACTTGCAGA	TGGTACTCCAGAAGACCAGAGG
IL-1β	GGGCCTCAAAGGAAAGAATC	TTGCTTGGGATCCACACTCT

MCP, monocyte chemoattractant protein; KIM, kidney injury molecule; TNF, tumor necrosis factor; IL, interleukin.



▶ **Fig. 1** Effects of exhaustive running exercise and macrophage depletion on macrophage infiltration in kidney of mice. (**a**) Histochemistry analysis of F4/80 and MCP-1 (brown; F4/80 and MCP-1 positive cells, original magnification × 200). A number of F4/80 positive cells are marked by arrows. G shows the renal glomeruli, RT shows renal tubules. (**b**) F4/80 positive cells. (**c**) F4/80 and (**d**) MCP-1 mRNA expressions in the kidney. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple comparisons. $^{**}P<0.01$, $^{*}P<0.05$. MCP, monocyte chemoattractant protein; Sed, sedentary; Ex, exercise.

▶ **Table 2** Effects of exhaustive exercise and macrophage depletion on renal function.

	Sed	Sed + Clodronate	Ex	Ex + Clodronate	Two-way ANOVA
BUN (mg/dL)	25.8 ± 1.1	26.0 ± 1.1	26.2 ± 2.3	21.8 ± 1.1	NS
CRE (μmol/L)	72.5 ± 3.1	71.2 ± 5.1	73.7 ± 5.1	75.7 ± 7.8	NS

Values are mean ± SE.; BUN, blood urea nitrogen; CRE, creatinine; Sed, sedentary; Ex, exercise.

Renal histology

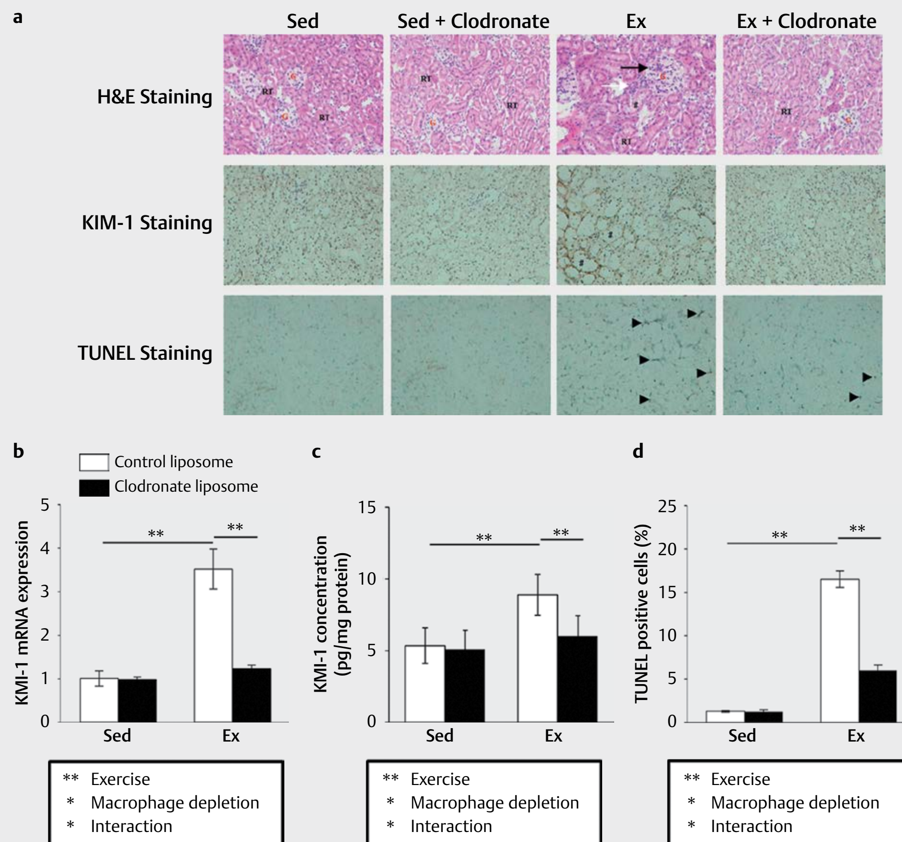
To evaluate renal damage, we performed H&E, KIM-1, and TUNEL staining. In H&E staining, the pathological changes were obvious in the exhaustive exercise group, manifested as congested and swollen glomeruli tubular dilatation, and nuclei infiltration. Compared with those of the exhaustive exercise group, the kidneys of the exhaustive exercise with macrophage depletion group showed far fewer histological abnormalities: fewer congested and swollen glomeruli, less tubular dilatation, and less nuclei infiltration were observed (▶ **Fig. 2a**).

KIM-1 is a marker of kidney injury. Immunohistochemical staining showed that KIM-1 expression in the exercise with control liposomes group was increased compared with the sedentary with control liposomes group (▶ **Fig. 2a**). On the other hand, increased KIM-1 expression was suppressed in the exercise with clodronate group (▶ **Fig. 2a**). Consistently, while KIM-1concentration and mRNA level was significantly increased in the exercise with control liposomes group compared with the sedentary with control liposomes group, injection of clodronate liposome reduced it (▶ **Fig. 2b, c**).

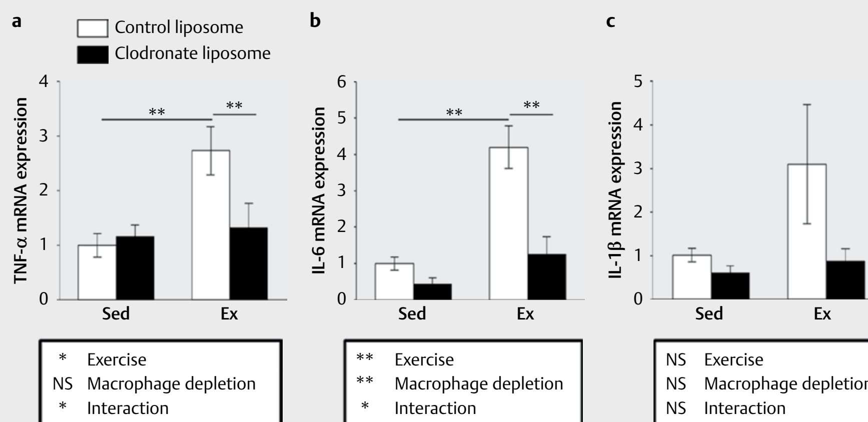
The TUNEL positive cells, a marker of cell apoptotic death, in the sedentary with control liposome group were 1.3%, whereas the exhaustive exercise with control liposome group showed 16.5% TUNEL positive cells. However, the percentage of TUNEL positive cells in the exhaustive exercise with clodronate liposome group was significantly lower than that observed in the exhaustive exercise with control liposomes group (▶ **Fig. 2d**).

Levels of inflammatory cytokines in the kidney

The effect of exhaustive exercise and macrophage depletion on the levels of inflammatory cytokines in the kidney 24 h after exercise was assessed with the expression levels of TNF-α (▶ **Fig. 3a**), interleukin (IL)-6 (▶ **Fig. 3b**), and IL-1β (▶ **Fig. 3c**) mRNA. While the expression levels of TNF-α and IL-6 were increased by exercise, they were ameliorated with clodronate (▶ **Fig. 3a, b**). The changes in



▶ **Fig. 2** Effects of exhaustive running exercise and macrophage depletion on kidney injury in mice. (**a**) H&E, KIM, and TUNEL staining of kidney sections (original magnification × 200), black arrow shows location of glomerular congestion and swelling, white arrow shows nuclear infiltration, and # shows tubular dilation. A number of TUNEL positive cells are marked by arrowheads. G shows the renal glomeruli, RT shows renal tubules. (**b**) (**c**) KIM-1 mRNA expression and concentration of kidney, (**d**) the percentage of TUNEL positive cells. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple comparisons. $^{**}P<0.01$, $^{*}P<0.05$. KIM, kidney injury molecule; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling; Sed, sedentary; Ex, exercise.



▶ **Fig. 3** Effects of exhaustive exercise and macrophage depletion on the expressions of inflammatory cytokines in the kidney. (**a**) TNF-α, (**b**) IL-6, and (c) IL-1β mRNA expression in the kidney. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple comparisons. $^{**}P<0.01$, $^{*}P<0.05$. Sed, sedentary; Ex, exercise; TNF, tumor necrosis factor; IL, interleukin.

the expression of IL-1β were similar, and there was no significant difference (▶ **Fig. 3c**).

Discussion

Endurance exercise induces acute renal damage [6], but the precise mechanisms are not well known. Clodronate liposomes are widely used to deplete macrophages [13]. In this study, clodronate liposomes were administered intraperitoneally in order to elucidate the role of macrophages in acute renal damage induced by endurance exercise in mice.

Infiltration of inflammatory cells is commonly seen in acute kidney injury by various causes [14]. In the ischemia/reperfusion model, it was reported that macrophage infiltration was observed in 24 h post-reperfusion [15]. On the other hand, after exhaustive exercise, infiltration of immune cells may occur in H&E staining [16]; however there are no reports that actually evaluate this. In this study, F4/80 positive cells determined by immunostaining and F4/80 mRNA expression level were significantly increased in the kidney 24 h after exhaustive exercise, and both were suppressed by clodronate liposomes. Thus, we showed that macrophage infiltration was observed after exhaustive exercise in our model and that we successfully achieved macrophage depletion by clodronate liposomes.

MCP-1 is a chemokine that belongs to the CC chemokine family. MCP-1 is expressed in many types of cells and predominantly recruits monocytes and macrophages to the sites of inflammation, and CC chemokine receptor type 2 (CCR2) is a receptor for MCP-1 and expressed on macrophages. Furuichi et al. reported that in the mice model of ischemia/reperfusion, macrophage infiltration was decreased in CCR2 knockout mice compared with the wild type [17]. It is also reported that MCP-1 expression was decreased with clodronate pretreatment in the ischemia/reperfusion model in the kidney [12]. Our immunohistologic study showed that the expression of MCP-1 in the exercise with control liposomes group was increased primarily in the tubular epithelial cells compared with the sedentary with control liposomes group after the exhaustive exercise, and the increase was suppressed by clodronate liposomes. However, there was no significant MCP-1 expression in glomeruli in our model. It is suggested that the increased MCP-1 expression by exhaustive exercise contributes to macrophage infiltration. In our model, the levels of mRNA expression of MCP-1 in the kidney were not significantly altered with the exhaustive exercise. We used total renal tissue for RNA extraction; therefore, it is possible that the changes only in the tubules were not sufficient to detect the significant difference by quantitative RT-PCR.

In our study, we determined that acute renal damage was induced by exhaustive exercise on the grounds that histological damage, KIM-1, and apoptosis were increased, although in our data renal function assessed by the levels of BUN and creatinine 24 h after the exercise was not significantly altered. In the previous study, the levels of BUN and creatinine were increased in 6 h and returned to normal in 24 h after exhaustive swimming [7]. We also found that BUN increased significantly immediately after exercise in our exhaustive exercise model, but fell to baseline after 24 hours (data not shown), suggesting that they might have increased earlier. However, these elevations might not have been observed because we sampled 24 hours after exercise to assess macrophage infiltration and inflammation in the kidney. We observed pathological changes as well as the elevated expression of KIM-1 in the kidney, and the increase was ameliorated with clodronate. The increased expression of KIM-1 recently emerged as a marker of acute kidney injury [18]. In addition, it was reported that KIM-1 expression was increased in the renal proximal epithelial cells in the post-ischemic kidney [19]. Apoptosis in tubular epithelial cells is also an important feature of acute kidney injury [20]. In previous studies, apoptosis in tubular epithelial cells was induced by endurance exercise [6] and exhaustive swimming [7]. In the present study, while increased TUNEL positive cells were observed in kidney, which demonstrated that apoptosis was induced by exhaustive exercise, it was ameliorated by macrophage depletion. It was reported that KIM-1 reduced high glucose-induced apoptosis in renal tubular epithelial cells, thus a direct relationship between KIM-1 and apoptosis has been suggested. Therefore, our data strongly suggest that acute renal damage by exhaustive exercise is attenuated when macrophage infiltration is blocked.

Macrophages are recruited into the inflamed tissues and contribute to tissue damage and inflammation via secretion of inflammatory cytokines [8]. In humans, the plasma concentrations of pro-inflammatory cytokines appear elevated after prolonged exercise [2, 21]. Moreover, protein expression of TNF-α was elevated in the kidney after exhaustive exercise in rats [7]. Interestingly, several studies reported that substances with anti-inflammatory properties were effective in suppressing acute renal damage following exhaustive exercise [6, 7]. Therefore, it is possible that acute renal damage by exhaustive exercise is influenced by mediators released from activated macrophages, including pro-inflammatory cytokines. In this study, we quantified mRNA expression levels of TNF-α, IL-6, and IL-1β to assess the inflammatory responses in the kidney. Their expression levels were increased in the ischemia/reperfusion model in mice [15], and these changes were suppressed by macrophage depletion [12]. Similarly, the levels of inflammatory cytokines were increased by exhaustive exercise but ameliorated by macrophage depletion in this study. Therefore, macrophage infiltration is likely to be a primary cause of local inflammation in the kidney following exhaustive exercise. This study also showed that the alteration in pro-inflammatory cytokine mRNA levels in the kidney was similar to the altered pattern of KIM-1 mRNA expression, protein concentration, and TUNEL positive cells. Therefore, induction of inflammation by macrophage infiltration may play a key role in renal damage following exhaustive exercise.

Interestingly, it was reported that there are different types of macrophages [22]. M1 macrophages predominantly produce pro-inflammatory cytokines such as TNF-α, whereas M2 macrophages produce anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist [23]. In acute kidney injury induced by ischemia/reperfusion, M1 macrophages are recruited into the kidney in the first 48 h, and M2 macrophages predominate at later time points and contribute to tissue repair [24]. In the present study, testing was carried out only at 24 h post-exercise. It is assumed that M1 macrophage depletion ameliorated the acute kidney damage induced by exhaustive exercise. Studies are needed to test different time points, and the roles of different macrophage phenotypes are needed to elucidate further the pathogenesis of acute renal dam-

age induced by exhaustive exercise. Immune cells other than macrophages have been reported to be involved in hypoxia/reperfusion-induced acute kidney injury [8]. It is reported that neutrophil depletion improved acute kidney injury induced by hypoxia/reperfusion and decreased inflammatory cytokines [25]. Recently, lymphocytes have also been shown to contribute to acute kidney injury [26]. How immune cells other than macrophages affect exercise-induced acute renal damage also needs further investigation.

In summary, exhaustive exercise caused inflammatory responses, apoptosis, and acute renal damage in mice, and these changes were attenuated with macrophage depletion by clodronate liposomes. This study has demonstrated that macrophages play a significant role in exhaustive exercise-induced acute renal damage.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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