Original Article

High-intensity exercise impairs intestinal barrier function by generating oxidative stress

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The intestine functions as a barrier preventing the entry of extrinsic factors into the body. This barrier function is disrupted by oxidative damage along with an impaired mucosal layer. Excessive exercise can generate oxidative stress in the intestinal tissue; however, the effect of exercise-induced oxidative stress on intestinal permeability is unclear. In this study, we examined the involvement of oxidative stress in barrier function of the ileum of mice following high-intensity exercise. Male ICR mice (12-week-old) were divided into sedentary and exercise groups. Mice in the exercise group underwent a single bout of treadmill running, and the ileum was collected for histological and biochemical analyses. Plasma fluorescence intensity level after oral administration of fluorescein isothiocyanate-dextran gradually increased until 30 min after exercise in response to intensity of exercise. Relatively high levels of oxidative proteins and low level of claudin-1, a tight-junction protein, were observed in the exercise group. Treatment with a xanthine oxidase inhibitor suppressed exercise-induced increases in intestinal permeability. Moreover, excessive exercise training for two weeks led to relatively high intestinal permeability at rest. These results suggest that high-intensity exercise increases intestinal permeability and tight junction damage, which may be mediated by oxidative stress.

***Key Words*: intestinal permeability, tight junction, high-intensity exercise, oxidative stress**

Habitual exercise reduces the risk of noncommunicable diseases, such as type 2 diabetes, cardiovascular diseases, and cancer,[1–3] by improving metabolic and immune functions and the cardiovascular system. Exercise benefits the gastrointestinal organs, such as profile of the microbiota, hormone secretion, and bowel movement.[4–6] Unlike moderate-intensity exercise, high-intensity exercise frequently causes oxidative stress, leading to metabolic impairment and adaptation failure.[7,8]

In skeletal muscle tissues, reactive oxygen species (ROS) are generated by various enzymes, such as NADPH oxidase, xanthine oxidase (XO), and those involved in mitochondrial electron transport during and after exercise.[9] During exercise, blood is primarily supplied to skeletal muscles in an intensity-dependent manner, while compensatory blood flow is reduced to the internal organs, particularly the liver, kidneys, and intestines.[10] Blood flow recovers during the resting period after exercise, resulting in a rapid increase in oxygen thrust. This ischemia/reperfusion-like stimulus increases the activity of XO and induces ROS production. Such oxidative stress may damage internal organs, particularly intestinal mucosal layer that is susceptible to oxidative damage.[11]

The intestine plays an important role in digesting and absorbing nutrients and in defense and immunity by preventing the entry of extrinsic factors into the body. Tight junctions between intestinal epithelial cells act as a barrier. Several proteins, including claudins, occludin, and zonula occludens, contribute to the maintenance of tight junctions and prevent the entry of bacteria, endotoxins, and antigens into the circulation via the paracellular route.[12] However, when the intestinal barrier function is compromised, extrinsic factors can easily enter the bloodstream and elicit inflammatory response and metabolic disturbance, which is referred to as a leaky gut.[13] It is implicated in intestinal pathologies such as inflammatory bowel disease, necrotizing enterocolitis, and celiac disease.[12] Various dietary factors and physical stress can cause leaky gut.[12,14] Circulating lipopolysaccharide and inflammatory factors are frequently found in athletes,[15] suggesting the development of a leaky gut during high-intensity exercise. However, the effects of high-intensity exercise on intestinal permeability and the involvement of oxidative stress remain unclear. In this study, we examined the involvement of oxidative stress on intestinal barrier function in the ileum of mice following high-intensity training.

Materials and Methods

Animals and experimental design. This study complied with the principles and guidelines of the Japanese Council on Animal Care and was approved by the Committee for Animal Research of Kyoto Prefectural University (KPU030410R). ICR mice (aged 10 weeks) (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were housed for two weeks in an air-conditioned (23 ± 2°C) room with a 12/12 h light/dark cycle.

First, to test the effect of acute exercise, mice were divided into sedentary and exercise groups. In the exercise groups, mice underwent a single bout of treadmill running at 30 m/min for 30 min and were dissected at 15, 30, and 60 min after exercise (Supplemental Fig. 1A*). To test the effect of exercise intensity, mice were further divided into three groups including sedentary, middle-intensity exercise (25 m/min for 30 min), and high-intensity exercise (32 m/min, inclined at 10° for 30 min) groups. Small intestine and blood samples were collected at each time point and used for further evaluation. We analyzed ileum intestine that shows lower barrier function compared to duodenum and jejunum[16] and has been used as a typical target of the barrier examination.[17,18] To test the effect of oxidative stress, mice were divided into sedentary, exercise, and XO inhibitor groups. Mice in the exercise and XO inhibitor groups were subjected to treadmill exercise at 30 m/min for 30 min. Thirty minutes before exercise, mice were administered allopurinol (160 μg/g body

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weight) or saline by gavage. Thirty minutes after exercise, the ileum and blood were collected for further evaluation.

Second, to test the effect of training, mice were divided into sedentary and exercise training groups. In the exercise training group, mice underwent treadmill training five times per week for 2 weeks, with gradually increasing running time and speed from 10 min at 10 m/min to 60 min, inclined at 10° at 32 m/min, according to our previous study.[8] At 24 h after the last bout of exercise, the ileum and blood were collected, and used for further evaluation (Supplemental Fig. 1B*).

Intestinal permeability test. After starvation for 4 h, mice received a 3.2% fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, St. Louis, MO) by gavage at 50 µl/10 g body weight. Blood samples were collected at each time point after exercise, and plasma FITC fluorescence intensity level was measured.

Immunohistochemistry. The ileum was cut and immersed in 10% formalin for fixation. Serial 8-µm thick transverse sections of the intestine tissue were prepared and mounted on silanized slides. One section was stained with hematoxylin and eosin. The sections were observed using a microscope (AE2000; Shimadzu Rika Co., Kyoto, Japan). The length of the intestinal wall was the average value of five locations selected at equal intervals. The remaining sections were washed with Histo-Clear (Cosmo Bio Co., Ltd., Tokyo, Japan) and deparaffinized. After rehydration, the sections were incubated with L.A.B. Solution (Cosmo Bio Co., Ltd.) for antigen retrieval. Then, the sections were blocked in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and incubated overnight at 4°C with primary antibodies against claudin-1 (Thermo Fisher Scientific, Waltham, MA) and occludin (Proteintech, Rosemont, IL). The sections were washed in PBS, incubated with goat anti-rabbit IgG (Thermo Fisher Scientific), and mounted using an antifade mountant (Thermo Fisher Scientific). The sections were observed using a fluorescence microscope (BZ-X800; Keyence, Osaka, Japan). Images were acquired following a unified condition, and the mean fluorescence intensity of the epithelium was quantified using ImageJ software (National Institute of Health, Bethesda, MD).

Protein extraction and immunoprecipitation. Proteins were extracted from samples of the small intestine using CelLytic-MT Lysis/Extraction Reagent (Sigma-Aldrich). Immunoprecipitation was performed using anti-claudin-1 antibody (Abcam, Cambridge, UK). Briefly, protein G agarose beads (Cytiva, Marlborough, MA) were mixed with anti-claudin-1 antibody and incubated with gentle rotation. After incubation, agarose beads were washed with Tris-buffered saline. The bead-antibody complex was again incubated at 4°C overnight under gentle rotation. Bound proteins were eluted from the beads by boiling in sodium dodecyl sulfate buffer.

Western blotting. Whole tissue protein and immunoprecipitates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and MagicMark XP Western Protein Standard (Thermo Fisher Scientific) was used for determining the molecular mass of proteins. The separated proteins were transferred onto nitrocellulose membranes, that were then incubated with primary antibodies against claudin-1 (Abcam) and hexanoyl-lysine adduct (HEL).[19] Protein bands were detected by chemiluminescence (Luminograph I; Atto Co., Ltd., Tokyo, Japan) and band densities were analyzed using ImageJ software.

Real-time polymerase chain reaction (PCR). Total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque Inc., Kyoto, Japan). After reverse transcription, quantitative PCR was performed using a Light Cycler 96 Real-Time PCR system (Roche Life Science, Penzberg, Germany) with TaqMan PCR Master Mix and TaqMan primers [*claudin-1*, ID Mm01342184_m1; *occludin*, ID Mm00500910_m1; *tumor necrosis factor α* (*TNFα*), ID Mm00443258_m1; *interleukin-1β* (*IL-1β*), ID Mm00434228_m1; *interleukin-6* (*IL-6*), ID Mm 00446190_m1; and *β-actin*, ID Mm006078939_s1] (Thermo Fisher Scientific) or with SYBR Green PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and primers (*Mucin-2*: forward, 5'-GAAGCCAGATCCCGAAACCA-3'; reverse, 5'-GAATCGGTA GACATCGCCGT-3', and 18S rRNA: forward, 5'-GCCGCTAGA GGTGAAATTCTTG-3'; reverse, 5'-CATTCTTGGCAAATG CTTTCG-3') (Greiner Bio-One Co. Ltd., Tokyo, Japan). Quantification cycle (Cq) values were determined using Light Cycler Software ver. 4 (Roche Life Science), and relative gene expression was quantified using the comparative Cq method, with *β-actin* or *18S rRNA* as the reference genes.

Statistical analysis. Data are presented as mean ± SE. All statistical analyses were performed using Statistical Package for Social Sciences ver. 22 (IBM, Armonk, NY). Differences between groups were evaluated using one-way analysis of variance (ANOVA) or unpaired *t* test. If ANOVA indicated a significant difference, the Tukey–HSD test was used to determine the significance of differences between means. A p value <0.05 was considered statistically significant.

Results

Acute high-intensity exercise increases intestinal permeability and damage. We examined the effect of exercise on intestinal permeability using FITC-dextran. Plasma FITC level elevated at 15 min after exercise, peaked at 30 min, and recovered to that of the sedentary state at 60 min (Fig. 1A). The FITC level at 30 min after exercise was markedly elevated in the high-intensity group, estimated as the exhausted exercise intensity above the lactate threshold, i.e., more than 70% of maximum oxygen uptake (Fig. 1B); however, this elevation was not found in the middle-intensity group.

Intestinal damage was histologically examined under leaky gut conditions. The thickness of intestinal wall was lower in the exercise group than that in the sedentary group (Fig. 1C). The level of claudin-1, a tight junction protein, was markedly decreased by high-intensity exercise (Fig. 1D) while the level of occludin, another major tight junction protein, tended to be lower in the exercise groups ($p = 0.0998$). (Fig. 1E).

Intestinal permeability is mediated by oxidative stress. To verify the role of oxidative stress in altering intestinal permeability, we analyzed oxidative modification of proteins in the intestine. Protein modification by a lipid peroxide, HEL, was higher in the exercise group than that in the sedentary group (Fig. 1F); specifically, a band near 70 kDa with high intensity was found in the exercise group. Furthermore, HEL modification of claudin-1 was detected in the immunoprecipitation assay (Fig. 1G). Further experiments using an XO inhibitor, allopurinol, were conducted to examine the effect of oxidative stress on exercise-induced leaky gut. Administration of allopurinol before exercise suppressed the increase in plasma FITC level induced by high-intensity exercise (Fig. 1H).

Intestinal permeability and damage in high-intensity exercise training. The effect of high-intensity exercise training for two weeks was examined on intestinal permeability. To avoid acute effects of the final exercise session, plasma samples and tissues were collected on the next day after exercise and analyzed. Plasma FITC level tended to be higher in the training group than that in the sedentary group ($p = 0.0527$) (Fig. 2A). In contrast to the results of acute exercise experiment, histochemical analysis showed that the length of intestinal wall was similar between groups (Fig. 2B). The mRNA level of *mucin-2* tended to be higher in the training group than that in the sedentary group ($p = 0.0752$) (Fig. 2C). Protein and mRNA levels of claudin-1 were unchanged in the two groups (Fig. 2D and F). Although occludin protein level was not markedly different between groups

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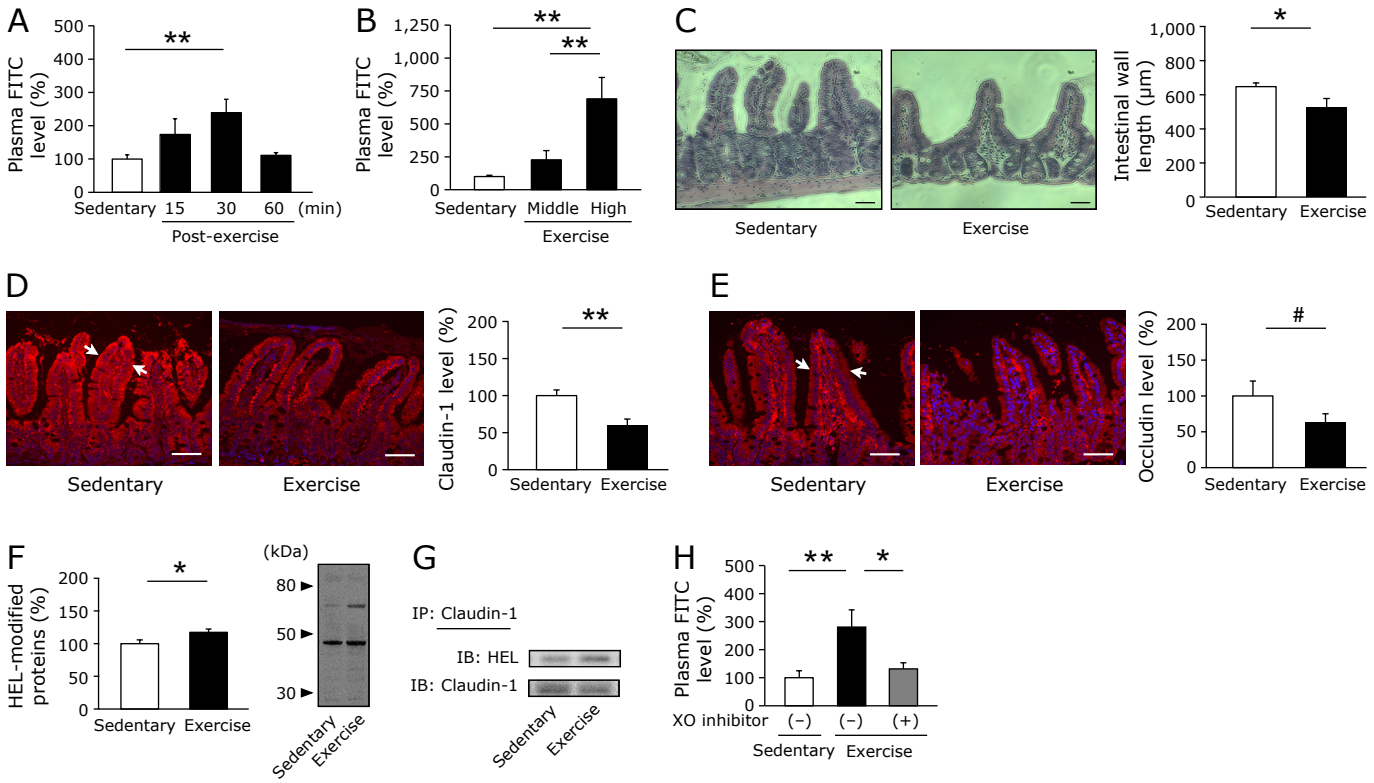

Fig. 1. Intestinal barrier function parameters after acute high-intensity exercise. The levels of plasma fluorescein isothiocyanate (FITC) level at 15, 30, and 60 min after exercise (A), and at 30 min in the middle-intensity (25 m/min for 30 min) and high-intensity (32 m/min, incline 10° for 30 min) exercise groups (B). Hematoxylin and eosin staining and intestinal wall length (C) in the ileum intestine. Bar, 100 μm. Immunofluorescence staining of claudin-1 (D) and occludin (E). Bar, 50 μm. The levels of HEL-modified proteins (F) and detection of HEL-modified claudin-1 in the ileum (G). The levels of plasma FITC level in sedentary, exercise, and exercise with xanthine oxidase inhibitor groups (H). Values are expressed as mean ± SE (n = 3–6). $^{\#}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$.

(Fig. 2E), mRNA level of *occludin* was higher in the training group than that in the sedentary group (Fig. 2F). HEL modification of proteins did not change in either group although several intense bands were observed (Fig. 2G). The mRNA level of *TNFα* was lower in the training group than that in the sedentary group ($p = 0.0653$) (Fig. 2H). The mRNA levels of *IL-1β* and *IL-6* were not different between groups (Fig. 2H).

Discussion

We observed that running exercise elevated intestinal permeability in an intensity-dependent manner. This elevation transiently peaked at 30 min after exercise and recovered to the sedentary level at 60 min. Furthermore, 2 weeks of exercise training resulted in elevated intestinal permeability at the resting state on the next day after last exercise. Consistent with elevated permeability, histological observations indicated decreased length of the intestinal wall length and disrupted tight-junction proteins after exercise. Previously, various indices related to intestinal damage, such as circulating parameters of permeability and endotoxemia, impaired appetite, extended intestinal transit, and nutrient malabsorption, have been found to be associated with intensity and duration of exercise;[20,21] these intestinal events have been suggested to be increased above 70% of the maximum oxygen uptake, i.e., under a high-intensity condition.[15] Our observations provide detailed information on intestinal damage caused by high-intensity exercise.

Oxidative stress can impair barrier function via oxidation of intestinal components. During exercise, blood is primarily supplied to the skeletal muscles, which accounts for the reduced blood flow in the intestine.[10] After exercise, XO and hypoxanthine react with oxygen delivered during the recovery of blood flow, thereby leading to ROS generation.[11] Therefore, the intestine may be a source of ROS after a single bout of exercise. We found that oxidative modification of proteins increased 15 min postexercise. Although claudin-1 modified by lipid peroxidation may be involved in disrupting the tight junction, further studies are needed to clarify the mechanism. In addition, treatment with an XO inhibitor suppressed elevated intestinal permeability after high-intensity exercise. These observations suggest that oxidative stress promotes exercise-induced intestinal permeability. Intestinal permeability is caused by the disruption of tight junctions formed by several proteins, including claudin and occludin. Relatively low claudin-1 levels were observed in the high-intensity exercise group. Claudins consist of 27 members,[22] which are required for intercellular tight junctions, physically occlude the paracellular space, create ion pores between cells, and maintain cell polarity.[23] Claudin-1 is widely expressed in the intestinal epithelium has barrier-forming abilities, and plays an important role in tight junction integrity.[24] *Claudin-1* knockout mice exhibit lethal dehydration in the skin and atopic dermatitis;[24,25] however, occludin deficiency is relatively less deleterious.[26] Previously, claudin-1 has been shown to be nitrated and ubiquitinated in fructose-fed rats, thereby leading to destruction of the tight junction.[27] Therefore, oxidative modification of claudin-1 may lead to decrease in protein contents, which results in destruction of the tight junction. Furthermore, as other possible factors, activated proteases and apoptosis also cause breakdown of cellular proteins and damage the intestinal wall.[28] Further studies are needed to

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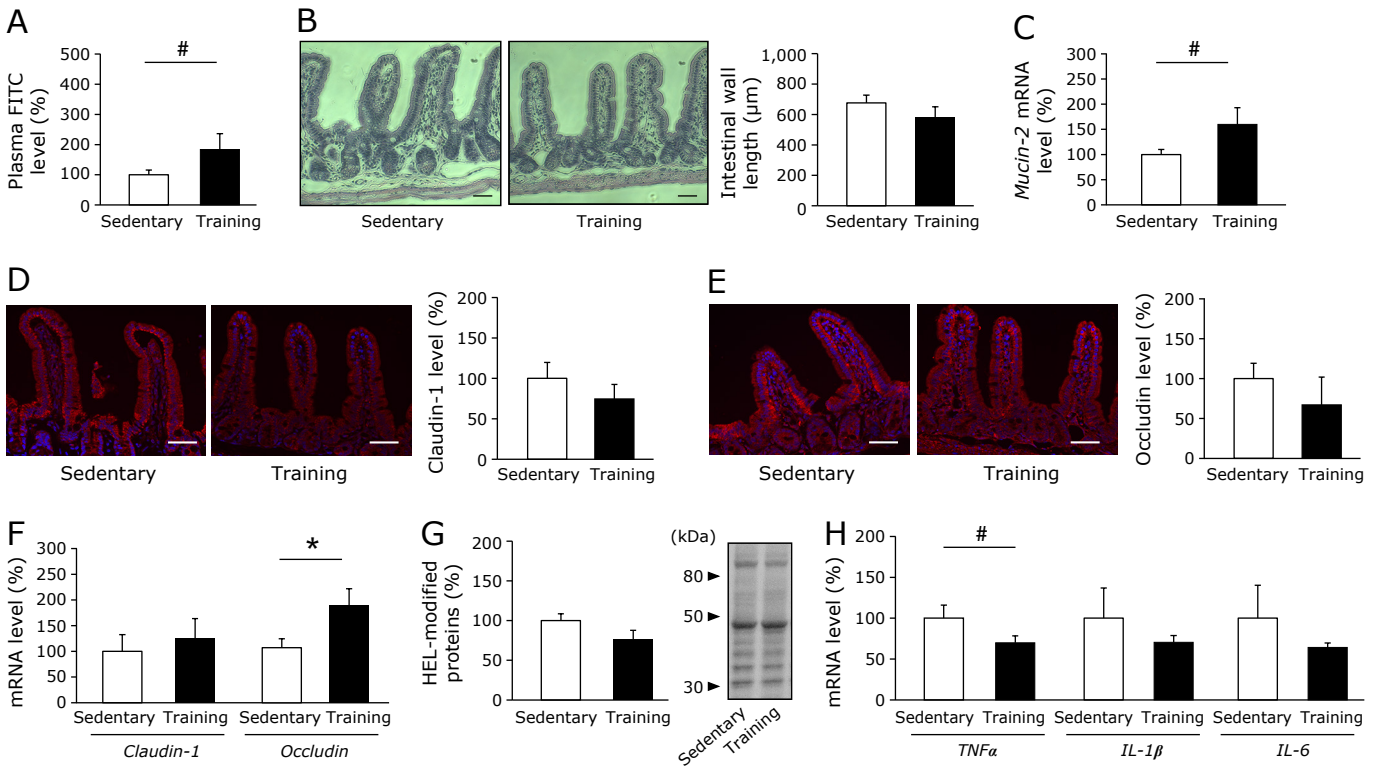

Fig. 2. Intestinal barrier function parameters after high-intensity exercise training. The level of plasma FITC level (A). Hematoxylin and eosin staining and intestinal wall length (B) in the ileum intestine. The mRNA level of *mucin-2* (C). Bar, 100 μm. Immunofluorescence staining of claudin-1 (D) and occludin (E). Bar, 50 μm. The mRNA levels of *claudin-1* and *occludin* (F) and levels of HEL-modified proteins (G). The mRNA levels of tumor necrosis factor alpha (*TNFα*), interleukin 1 beta (*IL-1β*), and interleukin 6 (*IL-6*) (H). Values are expressed as mean ± SE (n = 3–6). $^{\#}p<0.10$, $^{*}p<0.05$.

examine the involvement of apoptosis and protease on exercise-induced intestinal damage in the future.

Harmful substances can leak from the intestinal tract into the circulation through the paracellular route, resulting in increased intestinal permeability. Leakage factors can spread to the entire body via circulation and cause inflammation and metabolic impairments in other organs. Typically, a leaky gut impairs metabolic function, which is mediated by chronic low-grade inflammation.[29] We previously found that mice with leaky guts exhibited glucose intolerance,[30] which may be involved in low glucose uptake and mitochondrial dysfunction. Under these leaky-gut conditions, muscle fatigue and failure to metabolic adaptation take place easily. However, the leaky gut rapidly recovers after acute exercise; barrier dysfunction is transiently generated, which is associated with the short half-life of tight-junction proteins.[31] In this study, intestinal permeability recovered to the same level as that in sedentary mice after 60 min of exercise. Therefore, these harmful substances would not leak into the bloodstream to the extent that they could damage other tissues. In contrast, high-intensity exercise training caused relatively high intestinal permeability even on days without exercise, suggesting that the breakdown of the intestinal barrier function exceeds extent of recovery. In contrast to post-exercise condition, we observed unaltered claudin-1 and occludin contents in histological examination. Furthermore, the levels of HEL-modified proteins did not change after exercise training. On the day after exercise, oxidative proteins were already degraded, and new proteins were rapidly synthesized, allowing the recovery of protein contents and oxidative state. In contrast, mRNA level of *occludin* was higher in the training group than that in the sedentary group. Because the half-life of occludin is longer than that of claudin-1,[31] transcription may be still activated to complement protein loss. Mucus contributes to protection in intestinal homeostasis against mechanical, chemical, and biological attacks.[32] Particularly, the large gel-forming mucin-2, synthesized by intestinal goblet cells in the epithelial cell layer, is a major component of mucus.[33] Increased intestinal permeability and morphological defects in intercellular junctions have been observed in *mucin-2* knockout mice, a model of colitis.[34] We observed higher expression of mucin-2 in the training group, suggesting a recovery process of intestinal permeability after exercise. Furthermore, the training group showed lower expression of TNFα, a typical proinflammatory cytokine that can disrupt the intestinal barrier.[35] Thus, the intestinal components and events such as inflammatory factors, disruption of the tight junction, and mucus contents, suggest the recover process on the next day after exercise. Nevertheless, considering high intestinal permeability even at rest and athletes who repeat excessive exercise frequently have increased gastrointestinal symptoms and inflammatory cytokines,[15] the leakage factors can further cause negative effects on metabolic functions. Therefore, dietary prebiotics, probiotics, and antioxidants may counteract oxidative stress and inflammation generated in the intestine after high-intensity exercise. Further studies are required to examine detail exercise-induced leaky gut and dietary intervention in the future.

In conclusion, high-intensity exercise transiently impairs intestinal barrier. Higher oxidative proteins and lower tight junction proteins were observed in the exercised mice. Inhibition of ROS production suppressed the elevation of intestinal permeability induced by exercise. These results suggest that intestinal oxidative stress induced by high-intensity exercise may be involved in the disruption of tight junction proteins, leading to leaky gut condition.

Author Contributions

MT and WA designed and coordinated the study. MT, WA, and KM analyzed and evaluated the data. YKato, YKobayashi, and MK helped in the design and interpretation of data. MT and WA drafted the manuscript with inputs from other authors. All the authors critically reviewed and approved the final version of the manuscript.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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