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Exercise training attenuates adipose tissue fibrosis in diet-induced obese mice

Noriaki Kawanishi [a,*], Hiroyuki Niihara [b], Tsubasa Mizokami [b], Hiromi Yano [c], Katsuhiko Suzuki [a]

[a] Faculty of Sport Sciences, Waseda University, 2-579-15, Tokorozawa, Saitama 359-1192, Japan
[b] Graduate School of Sport Sciences, Waseda University, 2-579-15, Tokorozawa, Saitama 359-1192, Japan
[c] Department of Health and Sports Science, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki, Okayama 701-0193, Japan

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ABSTRACT

Tissue fibrosis, such as that which occurs in obesity, is associated with chronic inflammatory diseases. Although regular exercise reduces adipose tissue inflammation, the mechanisms regulating the effects of exercise on adipose tissue fibrosis are unclear. This study aimed to clarify whether exercise training attenuates adipose tissue fibrosis with consequent reduction of extracellular matrix including collagens. Male C57BL/6J (4-week old) mice were randomly assigned to four groups that received a normal diet (ND) plus sedentary ($n = 8$), an ND plus exercise training ($n = 8$), a high-fat diet (HFD) plus sedentary ($n = 12$), and an HFD plus exercise training ($n = 12$). Mice were fed the ND or HFD from 4 to 20 weeks of age. The exercise groups were trained on a motorized treadmill for 60 min/day, 5 times/week over the same period. Histological hepatic fibrosis detected by Sirius red and α-smooth muscle actin staining were attenuated in HFD exercise mice compared with HFD sedentary mice. mRNA levels of transforming growth factor-β and tissue inhibitors of metalloproteinase-1, major regulators of tissue fibrosis, were increased in HFD sedentary mice but were attenuated in HFD exercise mice. Similarly, adipose tissue from the HFD sedentary mice contained higher macrophages than adipose tissue from the ND mice, and this was also lowered by exercise training. These findings suggest that exercise training may be effective for attenuating adipose tissue inflammation in obesity.

1. Introduction

Obesity is associated with adipose tissue inflammation, which has been associated with the pathogenesis of chronic inflammatory diseases such as type 2 diabetes and non-alcoholic steatohepatitis (NASH) [1]. Particularly, the innate immune system regulated by macrophages is a key inflammatory response in adipose tissue, which contributes to insulin resistance [2]. Recently, it was demonstrated that adipose tissue fibrosis occurred in obesity along with inflammation [3]. Fibrosis is causally related to the pathogenesis of several chronic inflammatory diseases, including atherosclerosis and NASH [4]. Interestingly, recent evidence has indicated that adipose tissue fibrosis also leads to metabolic dysfunction. Khan et al. [5] reported that reduction of adipose tissue fibrosis resulted in improved insulin sensitivity in obese mice. Therefore, development of adipose tissue fibrosis is involved in insulin resistance of obesity.

Recent evidence has indicated that regular exercise prevents the development of chronic inflammatory diseases [6]. In obese patients, moderate exercise training reduces the plasma level of inflammatory cytokines and oxidative stress markers, and improves systemic inflammation [7]. Interestingly, exercise training is now considered to be a crucial mechanism for reducing adipose tissue inflammation. Recently, we reported that exercise training attenuates adipose tissue inflammation via suppression of macrophage infiltration in obese mice [8,9]. Similarly, we have shown that exercise prevents development of hepatic inflammation and fibrosis in obese mice [10]. In fact, exercise training attenuates hepatic fibrosis via reduction of activated hepatic stellate cell and collagen deposition in diet-induced obese mice [10]. However, the preventive effect of exercise on adipose tissue fibrosis and its regulatory mechanisms are unclear. We hypothesized that exercise training attenuates adipose tissue fibrosis with consequent reduction of extracellular matrix including collagens. We therefore examined the impact of exercise training on development of adipose tissue fibrosis in diet-induced obese mice.

Abbreviations: TIMP-1, tissue inhibitor of metalloproteinase-1; TGF-β, transforming growth factor-beta.

* Corresponding author. Fax: +81 4 2947 6753.
E-mail addresses: kawanishinoriaki@akane.waseda.jp (N. Kawanishi), h.niihara@akane.waseda.jp (H. Niihara), o-hisyo.w@akane.waseda.jp (T. Mizokami), yanohiro@mw.kawasaki-m.ac.jp (H. Yano), katsu.suzu@waseda.jp (K. Suzuki).

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2. Methods

2.1. Animals and diets

Male C57BL/6 mice (4 weeks of age) were purchased from Kiwa Laboratory Animals (Wakayama, Japan). Animals were housed 4 to a cage in a controlled environment with a 12 h light–dark cycle (lights on at 9:00 AM). The experimental procedures followed the Guiding Principles for the Care and Use of Animals of the Waseda University Institutional Animal Care and Use Committee (approval number 2012-A081). The mice were randomly assigned to 4 groups that received a normal diet (ND) plus sedentary ($n = 8$), a ND plus exercise training ($n = 8$), a high-fat diet (HFD) plus sedentary ($n = 12$), and a HFD plus exercise training ($n = 12$). The HFD comprised 60% of calories from fat, 20% from protein, and 20% from carbohydrate (D12492; Research Diets, New Brunswick, NJ, USA). The mice were fed the HFD from 4 to 20 weeks of age. The ND mice were fed a standard chow consisting of 10% of calories from fat, 20% from protein, and 70% from carbohydrate (D12450B; Research Diets). All groups had free access to food and water. The animals were weighed weekly, and food intake per cage (4 mice) was measured monthly.

2.2. Exercise training protocol

Exercise training was initiated when the mice were 4 weeks of age and continued for 16 weeks. Before the experiment, the mice were accustomed for treadmill running for 15 min once during acclimation period. The exercise training mice were placed on a motorized treadmill (Natsume, Kyoto, Japan) for 60 min/day (during the light phase), 5 days/week. The running speed was set at 15 m/min for the first 4 weeks and 20 m/min for the remaining 12 weeks. The mice were not subjected to electric shock during the treadmill sessions to avoid noxious stress. The non-exercised (sedentary) mice remained in their cages. The exercise-trained and untrained mice (20-weeks old) were sacrificed 3 days after the final exercise training session under light anesthesia with the inhalant isoflurane (Abbott, Tokyo, Japan). The epididymal adipose tissue was quickly removed, weighed, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Histological analysis

A piece of the epididymal adipose tissue was transferred to a plastic mold, covered with O.C.T. compound, and snap frozen by immersing in pre-cooled isopentane at −80 °C. Cryo specimens were stained by Sirius red, and Masson trichrome. Sirius red-staining positive areas were analyzed on four random low power (100×) fields/slide using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical staining was applied to frozen sections to examine expression of α-SMA and F4/80. The 8 μm serial sections were incubated in 4% paraformaldehyde for 7 min at 4 °C. Endogenous peroxidase was inactivated with 1% hydrogen peroxide in methanol for 30 min at 4 °C. α-Smooth muscle actin (Dako, Carpinteria, CA, USA) and F4/80 (Serotec, Kidlington, UK) primary antibodies were added in 1% BSA solution and sections were incubated overnight at 4 °C. Secondary anti-rabbit antibody was added in PBS buffer with normal mouse serum for 30 min at room temperature. Proteins were visualized using the Vectastain Elite ABC Kit (anti-rabbit, Vector, Burlingame, CA, USA) for 30 min at room temperature and further incubation was carried out with diaminobenzidine (DAB) chromogen.

Calculations of Sirius red-positive areas were performed by three independent observers, who were blinded to the diagnosis, and the average value for each section was calculated. The coefficient of variation for Sirius red-positive areas score was 14.7%.

2.4. Real-time quantitative PCR

Another piece of the epididymal adipose tissue was quickly immersed in RNAlater (Applied Biosystems, Carlsbad, CA, USA) and stored at −80 °C. Total RNA was extracted from the epididymal adipose tissue homogenate using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 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 to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed with the Fast 7500 real-time PCR system (Applied Biosystems) using the Fast SYBR Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of 10 min at 95 °C for denaturation followed by 40 cycles of 95 °C for 3 s and annealing at 60 °C for 15 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the housekeeping gene, and all data are represented relative to its expression (i.e., using standard curve methods) as fold change based on the values of the ND sedentary group. Specific PCR primer pairs for each studied gene are shown in Supplemental Table 1.

2.5. Statistical analyses

All data are expressed as means ± SEM. Statistical analyses were performed using SPSS V19.0. Body weight changes were analyzed using a general linear model analysis of variance (ANOVA) with repeated measures with Bonferroni post-hoc tests. The statistical significance of differences in histological analysis and mRNA expression was determined using two-way analysis of variance (ANOVA) with diet (ND or HFD) and exercise (sedentary or exercise training). When significant interactions were observed in any of the analyses, multiple comparisons were performed using Bonferroni post-hoc tests after one-way analysis of variance (ANOVA). The alpha level was set at $p < 0.05$.

3. Results

Fig. 1 illustrates the changes in body mass that occurred during the intervention. Using repeated measures analysis of variance (ANOVA), there were significant time, group, and time × group ef-

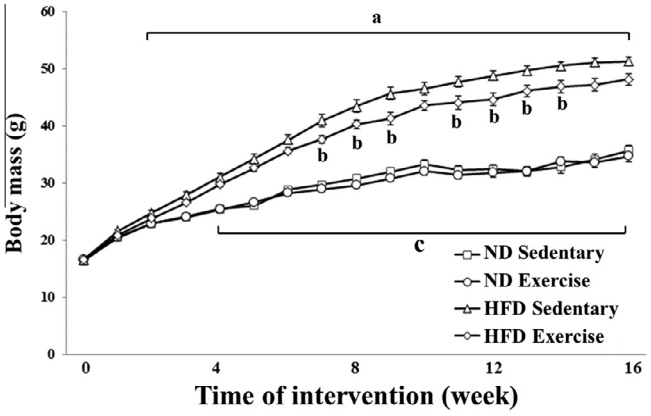

Fig. 1. Body mass across the intervention. Repeated measures analysis of variance (ANOVA) revealed significant time and time × group effects for body mass. [a]$p < 0.05$, different from ND sedentary, [b]$p < 0.05$, different from HFD sedentary, [c]$p < 0.05$, different from HFD exercise.

fects for weekly body mass. HFD sedentary mice were heavier than ND sedentary mice from weeks 2 to 16. HFD exercise mice were lighter than HFD sedentary mice from weeks 7 to 9, and weeks 11 to 14, although there was no significance difference in body mass at the end of the intervention (52.4 ± 1.6 and 48.1 ± 1.1 g for HFD sedentary and HFD exercise, respectively).

To determine whether exercise impacted adipose tissue fibrosis, we analyzed collagen deposition (Fig. 2). The Sirius red and Masson trichrome, which were used to stain collagen types 1 and 3, were low in ND sedentary mice. HFD markedly induced collagen staining in adipose tissue, as observed around individual adipocytes. However, exercise trained mice exhibited less collagen deposition in adipose tissue in response to a HFD (Fig. 2A). The Sirius red staining was calculated as the percentage of positive-stained areas per square millimeter in each section, and ANOVA revealed a significant diet × exercise interaction ($F_{1,28} = 32.96$, $p < 0.01$). Although the positive area was significantly greater in HFD sedentary mice than in ND sedentary mice, this was significantly lower in HFD exercise mice compared to HFD sedentary mice (Fig. 2B). Tissue fibrosis is regulated by increased activation of fibroblast and collagen deposition. Although activated fibroblasts are characterized by α-smooth muscle actin (SMA) expression, immunohistochemistry staining with α-SMA antibody detected activated fibroblasts. HFD induced activation of fibroblasts in adipose tissue. However, HFD exercise trained mice showed a marked suppression of activated fibroblasts, as seen by the reduction in the number of detected α-SMA positive cells (Fig. 2A). With respect to mRNA levels of collagen 1α and 3α, which are markers of tissue fibrosis, we found a significant diet × exercise interaction (collagen 1α mRNA; $F_{1,36} = 11.11$, $p < 0.01$, collagen 3α mRNA; $F_{1,36} = 24.29$, $p < 0.01$). Post-hoc comparisons revealed that collagen 1α and 3α mRNA levels were higher in HFD sedentary mice than in ND sedentary mice, and were significantly lower in HFD exercise mice than in HFD sedentary mice (Fig. 2C).

Transforming growth factor (TGF)-β and tissue inhibitors of metalloproteinase (TIMP)-1 are critical in driving fibrogenesis in adipose tissue. To investigate the mechanism of improvement of adipose tissue fibrosis by exercise, we measured TGF-β and TIMP-1 expression (Fig. 3). TGF-β and TIMP-1 mRNA levels in the adipose tissue varied as a diet × exercise interaction (TGF-β, $F_{1,36} = 11.74$, $p < 0.01$; TIMP-1, $F_{1,36} = 4.58$, $p < 0.05$). Post-hoc comparisons revealed that the levels of TGF-β and TIMP-1 mRNA in the

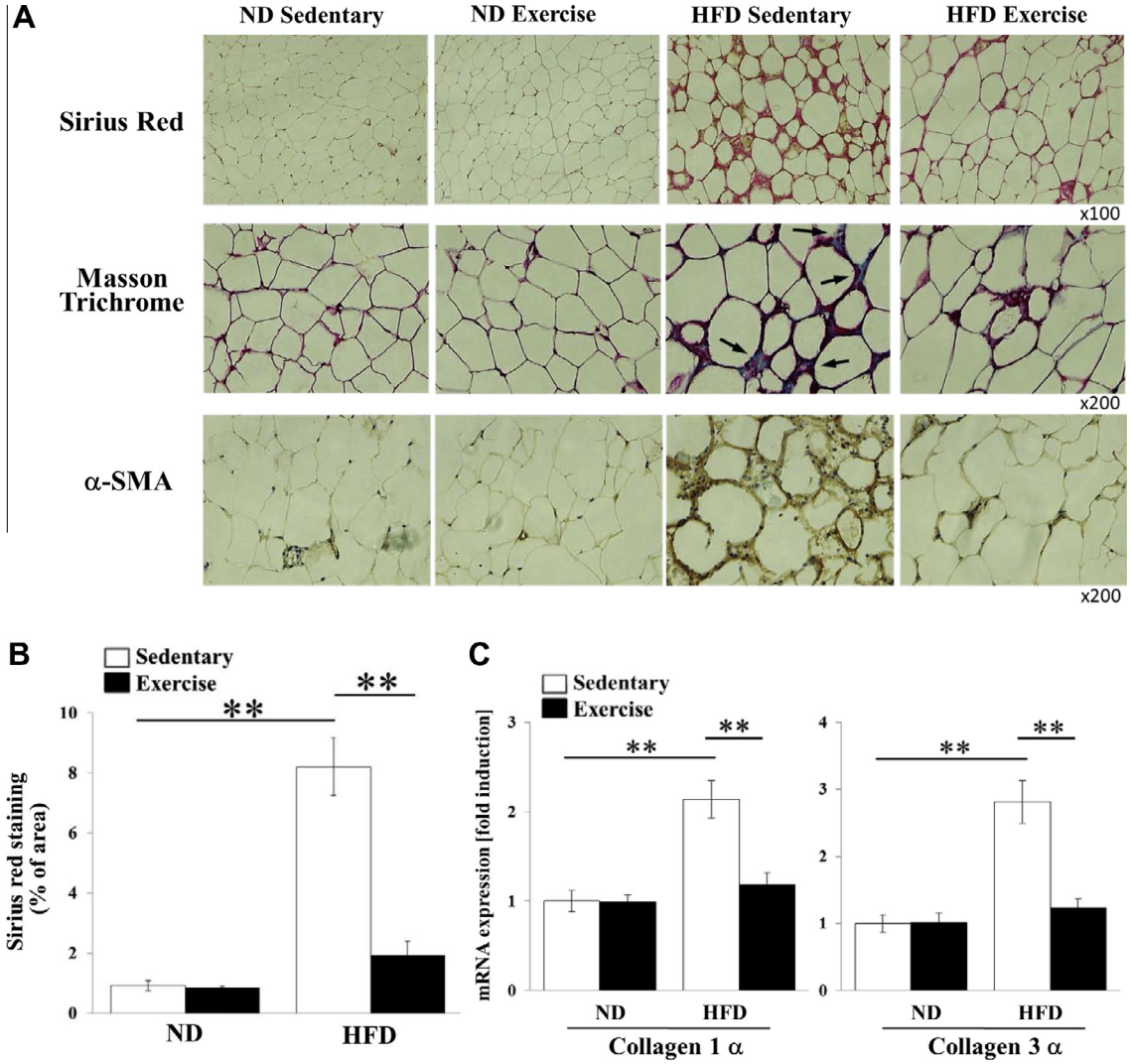

Fig. 2. Effect of exercise training on adipose tissue fibrosis in ND- and HFD-fed mice. (A) Sirius red (red; collagen fiber), Masson trichrome (arrowheads; collagen fiber), and α-SMA immunohistochemistry (brown; α-SMA positive cells) staining of epididymal adipose tissue sections. (B) The Sirius red-positive area. (C) mRNA expression levels of collagen 1α and collagen 3α in epididymal adipose tissue. Values represent means ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

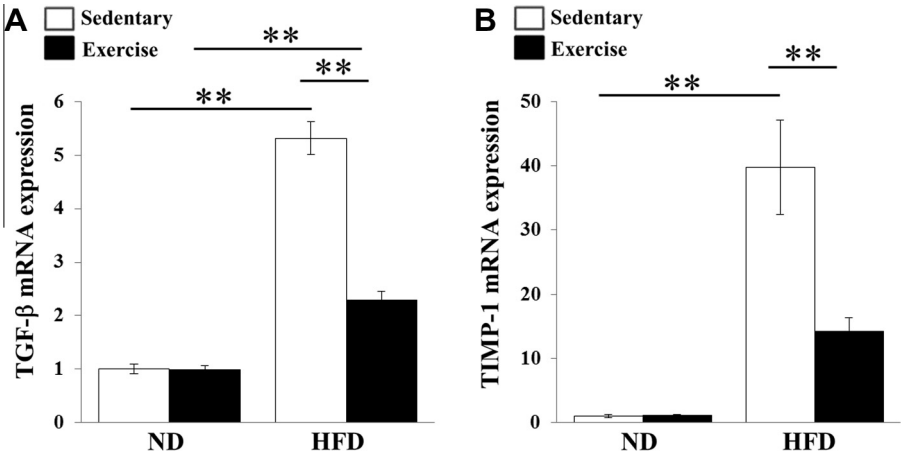

Fig. 3. Effect of exercise training on gene expression of fibrogenic markers in ND- and HFD-fed mice. The mRNA expression levels of TGF-β (A) and TIMP1 (B) in epididymal adipose tissue. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups, NS; not significant, $^{**}p < 0.01$.

HFD sedentary mice were higher than those in the ND sedentary and the HFD exercise mice (TGF-β, $p < 0.01$; TIMP-1, $p < 0.01$; Fig. 3).

Although TGF-β and TIMP-1 are the major regulators to mediate tissue fibrosis, these mediators are mainly produced by monocytes and macrophages. We investigated whether the attenuation of adipose tissue fibrosis response in exercise training was associated with suppression of macrophage infiltration. F4/80 immunohistochemistry, which was used to stain monocytes and macrophages, revealed the presence of greater number of macrophages in HFD sedentary mice around the adipocytes. However, the number of macrophages around adipocytes were markedly reduced by exercise training (Fig. 4A). We also found a significant diet × exercise interaction with respect to the mRNA level of F4/80 ($F_{1,32} = 7.96$, $p < 0.01$). Post-hoc comparisons revealed that the F4/80 mRNA level was significantly increased in the HFD sedentary mice compared to the ND sedentary mice and significantly lower in HFD exercise mice than in HFD control mice (Fig. 4B).

4. Discussion

Adipose tissue fibrosis is causally related to the pathogenesis of chronic inflammatory diseases such as type 2 diabetes [11]. Recently, it has been shown that visceral adipose tissue in obese humans has severe collagen deposition compared to non-obese humans [12,13]. Khan et al. reported that collagen-deficient mice have reduced adipose tissue fibrosis with improved insulin sensitivity, suggesting that adipose tissue fibrosis may be of central

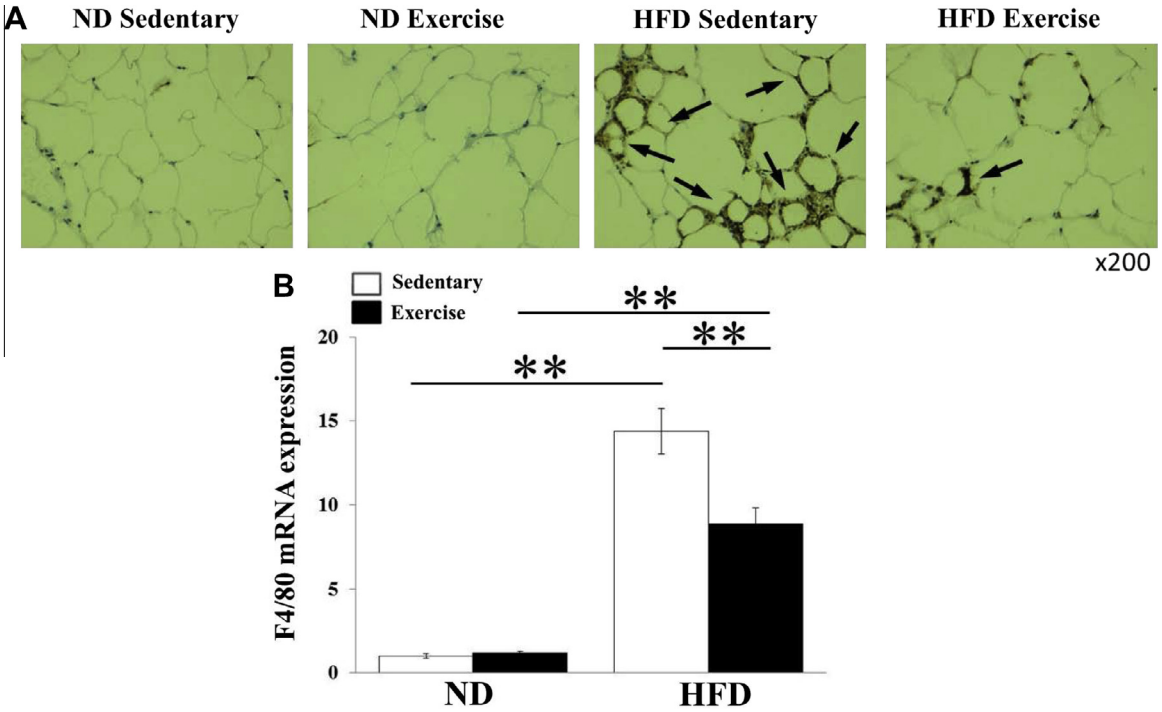

Fig. 4. Effect of exercise training on infiltration of macrophages into the adipose tissue of ND- and HFD-fed mice. (A) F4/80 immunohistochemistry (brown; F4/80-positive cells, arrowheads; crown like structure) staining of epididymal adipose tissue sections. (B) F4/80 mRNA levels in epididymal adipose tissue. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups. $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

importance for development of insulin resistance. Regular exercise reduces the risk of chronic inflammatory disease, because exercise has anti-inflammatory effects [6]. We have previously reported that exercise training attenuates adipose tissue inflammation in obese mice [8,9]. Exercise training also not only suppressed hepatic inflammation but also inhibited development of hepatic fibrosis [10]. However, whether exercise training attenuates adipose tissue fibrosis is unclear. In this study, we evaluated the effect of exercise training on obesity-induced adipose tissue fibrosis with changes in extracellular matrix.

Obesity induces collagen deposition into the visceral adipose tissue and leads to a development of adipose tissue fibrosis in mice and humans [14,15]. In fact, Michailidou et al. [16] reported that administration of a high-fat diet induces adipose tissue fibrosis via enhancement of collagen deposition. We have found that exercise training decreases collagen deposition in visceral adipose tissue of the diet-induced obese mice. Consistent with fewer collagen depositions, collagen 1 and 3α mRNA levels of adipose tissue were lower in HFD exercise training mice than HFD sedentary mice. Therefore, in obese mice, exercise training is likely to attenuate adipose tissue fibrosis by preventing collagen deposition.

Several studies to understand the molecular mechanism of tissue fibrosis development have shown that TGF-β is a key regulator [17]. In particular, TGF-β has been reported to be associated with increased release by visceral adipose tissue from obese patients [14]. Fibroblasts are activated by TGF-β, and then differentiate into α-SMA expressing fibroblasts to produce TIMP-1 [18]. Importantly, TIMP-1 plays a crucial role in the pathogenesis of tissue fibrosis [19]. Hellerbrand et al. [20] reported that, compared with wild-type obese mice, TGF-β-deficient obese mice exhibited reduced collagen deposition and activated fibroblasts in the liver. Therefore, TGF-β seems to be a critical cytokine mediating fibrosis via induction of activated fibroblasts. Consistent with other recent studies, we observed activation of fibroblasts in the adipose tissue of the HFD sedentary mice, but this was markedly suppressed by exercise training. Similarly, we found that levels of TGF-β and TIMP-1 mRNA were significantly reduced by exercise training. We also observed that TGF-β mRNA levels in adipose tissue were correlated with the area of collagen deposition. Therefore, exercise training may attenuate adipose tissue fibrosis by inhibiting the obesity-induced TGF-β up-regulation in obese mice.

Fibrosis is closely linked with innate immune responses. In obesity, macrophages exert paracrine actions on adipose tissue via production of several mediators [21]. Macrophages produce TGF-β, and induce extracellular matrix regulation in tissue [22]. Macrophages also produce matrix metalloproteinases (MMPs) and TIMP-1 by stimulation of inflammatory cytokines such as TNF-α [23]. Indeed, this study showed that the alteration in macrophage infiltration was similar to the altered pattern of TGF-β and TIMP-1 mRNA levels in the adipose tissue. Therefore, macrophages may play an important role in the balance between extracellular matrix formation and degradation in adipose tissue. Recent studies reported that macrophage depletion by clodronate liposomes reduced collagen deposition and activated fibroblasts in liver and kidney of fibrosis model mice [24,25]. Thus, reduction of the macrophages may be important for the therapy of tissue fibrosis. We observed that exercise training in obese mice reduced F4/80 positive cells, and attenuated F4/80 mRNA level in the adipose tissue. In this study, we have also found that F4/80 mRNA levels were correlated with the percentage of Sirius red positive-stained area. Therefore, suppression of macrophage infiltration by exercise training may be associated with an improvement of adipose tissue fibrosis in obese mice.

In summary, we have demonstrated that exercise training markedly attenuates the collagen deposition and activation of fibroblast, and reduces macrophage infiltration in visceral adipose tissue. Taken together, our results provide evidence that exercise training plays a critical role in reducing adipose tissue fibrosis by regulating the infiltration of macrophages.

Authors contribution

N.K. contributed to the conception and design of this study and to write the manuscript. N.K., H.N., T.M. and H.Y. performed the experimental work and analysis of the results. K.S. has primary responsibility for final content.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.004.

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