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ORIGINAL PAPER

Effects of exercise and diet composition on expression of MCP-1 and oxidative stress-related mRNA of adipose tissue in diet-induced obese mice

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ABSTRACT

The aim of the study was to analyze how the expression of MCP-1, HIF-1α, NOX2, ERK1, ERK2, and Mn-SOD mRNA, which are related to inflammation and oxidative stress and which can influence the accumulation of macrophage in obese adipose tissue, differed according to a high-fat diet, change of diet composition, and exercise. Obesity was induced using a high-fat diet (45% fat) for five weeks. This investigation analyzed how the change of diet composition for eight weeks and long-term exercise training affected the expression of mRNA in epididymal white adipose tissue. For the experiment, 56 four-week-old C57BL/6 mice were used. Their epididymal white adipose tissue was extracted and used in RT-PCR analysis to find the expression level of mRNA. A high-fat diet for 13 weeks showed a significant increase in the expression of MCP-1, HIF-1α, NOX2, and ERK1 mRNA in epididymal adipose tissue. Change in diet composition and exercise decreased the expression of MCP-1, HIF-1α, NOX2, and ERK1 mRNA. Particularly, the group combining a high-fat diet and exercise had a significant increase in the expression of Mn-SOD mRNA in epididymal adipose tissue; however, it showed a significant decrease in MCP-1, HIF-1α, and NOX2. These results suggest that the antioxidant effect and weight loss by exercise decreased inflammation and oxidative stress.

Keywords: diet-induced obesity, exercise, MCP-1, oxidative stress

INTRODUCTION

Obesity results from an excessive increase in adipose tissue due to an inactive lifestyle and increased high-energy food intake. It is one of the major causes of metabolic disorders such as diabetes, cardiovascular disease, hypertension, and hyperlipidemia, all of which contribute to the increasing mortality rate [1,2]. These metabolic disorders from obesity can cause a disorder of secreting excessive cytokine and chemokine produced in adipose tissue and hormones [3,4]. Also, the degree of obesity has a static correlation with MCP (monocyte chemoattractant protein)-1 and macrophage markers that can cause endocrine dysfunction [5].

Anoxia and oxidative stress have the possibility of influencing the increase of MCP-1 expression and the mobilization of macrophage. Recently, the results of studying obese animal models by ob/ob and KKAy as well as studies on high-fat diet proved that obesity causes hypoxia in white adipose tissue (WAT) [6-8], which in turn triggers the control disorder adipokine including MCP-1 due to the concomitant increase in oxidative stress [9,10]. The growth of reactive oxygen species (ROS) in adipose tissue is related to the increase of MCP-1 [11,12]. Also, it has been shown that NADPH (nicotinamide adenine dinucleotide phosphate) oxidase has a significant increase in the adipose tissue of obese people [10, 13]. Therefore, it is meaningful to find how the expression of HIF (hypoxia inducible factor)-1α caused by in the condition of hypoxia of adipose tissue and NOX (NADPH oxidase)2 a member of NADPH oxidase is related to the expression of MCP-1, and to analyze how HIF-1α and ERK (extracellular signal- regulated protein kinase)1/2, a transcription factor of ROS, influences the expression of MCP-1.

The most general method for preventing and treating chronic mild inflammation caused by obesity is to reduce weight. Research indicates that losing weight through diet and exercise decreases the inflow of macrophage into the adipose tissue. Previous investigations looked into the effect of weight

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loss on hypoxia as well as the effect of oxidative stress on the accumulation of macrophage [14]. However, research on other effects of exercise and change in diet composition without limiting food consumption/intake is scarce. In those studies, subjects with long term and regular exercise routine showed lower oxidative stress than those without exercise [15]. This was believed to be due to the increase of endogenous antioxidants, such as Mn-SOD (manganese superoxide dismutate) and GPX (glutathione peroxidase). Research investigating the antioxidant effects of exercise on reducing MCP-1, which induces the inflow of macrophages into adipose tissue, is insufficient.

Therefore, the purpose of this study was to analyze how a high-fat diet, diet composition change, and long term exercise on C57BL/6 mice influence the expression of MCP-1, HIF-1α, NOX2, ERK1&2, and Mn-SOD mRNA of WAT.

Table 1. Division of experimental mice

Group	Base	ND	HFD	HFD		ND	
				Non-Ex	EX	Non-Ex	EX
Number	8	8	8	7	7	8	8

Base: baseline, HFD: high fat diet, ND: normal diet, Non-EX; non exercise, EX: exercise

Table 2. Exercise protocol

Weeks	Warming up		Main exercise	
	Speed (m/min)	Time (min)	Speed (m/min)	Time (min)
1	8	5	18	30
2	8	5	18	40
3	8	5	18	50
4	10	5	18	60
5	10	5	18	60
6	10	5	22	60
7	15	5	22	60
8	15	5	22	60

METHODS

Animals for experiment

56 four-week-old C57BL/6 mice were use in this study. They were given a one-week adjustment period before the start of the experiment. Random sampling was used to assign the mice to different treatments. After the adjustment period, 8 mice were chosen to serve as a baseline group. Another 8 were given a normal diet for 5 weeks before their samples were taken (ND). From the remaining 40 mice with a high-fat diet, 8 were selected for the high-fat diet group (HFD). The rest 32 were treated with exercise and diet for 8 weeks and assigned to 4 different groups: with a high-fat diet and did not receive exercise treatment (HFD-Non-EX), with a high-fat diet and with exercise treatment (HFD-EX), with a normal diet and with no exercise treatment (ND- Non-EX), and with a normal diet and with exercise treatment (ND-EX). Groups of 45% high-fat diet and 10% normal diet could freely take food as well as drink water according to the characteristics of each group. During breeding, light and dark cycles were 12 hours per day, the temperature and the relative humidity of the breeding room were 24 ± 1℃ and about 60%, respectively, and four mice were bred per cage.

Exercise treatment

One week before the exercise treatment, adjustment exercise was carried out with the speed of 10-18m/min for 30 minutes for three days a week. Then, 8 weeks of exercise treatment was implemented with 35-65 minutes of treadmill per day for 5 days per week. The strength of the exercise used in obesity treatment was set at about 60-75% of maximum heart rate which was a modification of the preceding research [16]. More detailed information on the speed and times of the treadmill exercise are shown in Table 2.

Analysis method

The weight of the mice was measured using Dial-O-Gram® Balance at one week intervals prior to the experiment, and the degree of expression of MCP-1, HIF-1α, NOX2, ERK1/2, and Mn-SOD mRNA in epididymal adipose tissue were analyzed after diet and exercise treatment. To rule out the effect of acute exercise, sampling was taken 48 hours after the last treadmill exercise. Mice were put under general anesthesia by injection of sodium pentobarbital (40 mg/kg body weight) into the abdominal cavity, and a complete abdominal incision was carried out. Epididymal adipose tissue was extracted, quickly frozen (freeze-clamp) in liquid nitrogen, and stored at -70℃ until the next analysis.

Extraction of total RNA

Total RNA was ground for 30-60 seconds on the ice-using homogenizer after adding TRI reagent (Sigma-Aldrch, Inc., USA) 1ml into 50-100 mg tissue. Ground tissue chloroform 200 μl was added, vortexed and kept for 10 minutes at room temperature, and the liquid in the upper phase was separated using a centrifuge with 13,000 rpm at 4℃ for 15 minutes. Isopropanol 500 μl was added to the separated liquid in the

Table 3. Primer sequences

Gene	Primer Sequences
MCP-1	Forward 5'-ACTGAAGCCAGCTCTCTCTTCCTC-3' Reverse 5'-TTCCTTCTTGGGGTCAGCACAGAC-3'
HIF-1α	Forward 5'-GCACTAGACAAAGTTCACCTGAGA-3' Reverse 5'-CGCTATCCACATCAAAGCAA-3'
NOX2	Forward 5'-TTGGGTCAGCACTGGCTCTG-3' Reverse 5'-TGGCGGTGTGCAGTGCTATC-3'
ERK1	Forward 5'-CGGCTGAAGGAGTTAATCTT-3' Reverse 5'-GAAGGAGACAGGTAGGAGCAG-3'
ERK2	Forward 5'-ACCGTGACCTCAAGCCTTC-3' Reverse 5'-TGATCTGGATCTGCAACACG-3'
Mn-SOD	Forward 5'-CATTAACGCGCAGATCATGC-3' Reverse 5'-CCCAAAGTCACGCTTGATAGC-3'
GAPDH	Forward 5'-TGTGGATCTGACATGCCGC-3' Reverse 5'-GAATGGGAGTTGCTGTTGAAG-3'

Table 4. Comparisons of body weight among groups in obesity-induced period for 5 weeks (g)

Item	Base	ND	HFD	F(T)-value
BW	12.14 ± 0.34^{a}	23.83 ± 1.44^{b}	29.34 ± 3.20^{c}	$149.303^{\#\#\#}$
Intake/wks	-	2.50 ± 0.27	2.31 ± 0.22	1.220

Values are mean ± SD. $^{\#\#\#}(p < 0.001)$: significance among groups in one-way ANOVA. Values with different letters in each row are significantly different from each other($p < 0.05$)

upper phase and was centrifugally separated with 13,000 rpm at 4 ℃ for 10 minutes. All was removed except the pallet after the centrifugal separation. The remaining RNA pallet was cleaned in 70% alcohol and then dissolved in a water bath of 55-60℃ for 15 minutes by adding triple distilled water treated with DEPC. Total RNA concentration was calculated by measuring absorbance at 260nm using UV spectrophotometer. Later, polluted DNA and DNase which could be included in extracted RNA were removed using TURBO DNA-free^{TM}Kit. cDNA was synthesized by Total RNA using iScriptTM cDNA Synthesis Kit. Real-time PCR was carried out with a method of amplification iniQ5 Multicolor Real-Time PCR Detection System by mixing MCP-1, HIF-1α, NOX2, ERK1, ERK2, MnSOD, and a specific primer of GAPDH proposed in Table 4 with each enzyme reaction reagent 2×SYBR Green Supermix. After PCR, RNA expression levels of MCP-1, HIF-1α, NOX2, ERK1, ERK2, and Mn-SOD were quantified as a ratio toward GAPDH, a house keeping gene.

Data processing

All data were used in calculating the average and the standard deviation of each group using the SPSS17.0 statistics program. For verifying average differences among groups for measurable variables, one-way ANOVA was used. If there were significant differences among groups, Tukey's post hoc test was used. To verify the interaction between exercise and diet of treated groups, two-way ANOVA analysis was carried out. Statistical level of significance was set at less than 5% ($p < .05$).

RESULTS

Weight difference by a high-fat diet among groups

Normal diet groups and high-fat diet groups showed an increase in weight as the mice grew older. In the last week of the obese-induced period, the high-fat diet groups (HFD) showed a significant increase ($p < 0.001$) in weight compared to the normal diet group (ND)(Table 4). The HFD-Non-EX group, whose obesity was induced with a high-fat diet and later fed with another high-fat diet for 8 weeks, showed significantly higher weight than HFD-EX, ND-Non-EX, and the ND-EX groups; however, there was no difference in weight among the HFD-EX, ND-Non-EX and the ND-EX groups (Fig. 1).

Difference of mRNA expression in WAT by a high-fat diet

Analysis of how a high-fat diet influences mRNA expression related to the accumulation of macrophage in WAT of mice was carried out. In the expression of MCP-1 mRNA, there was no significant difference between the ND group and the Baseline group. The HFD group with a high-fat diet for five weeks, however, showed a significantly higher expression (p

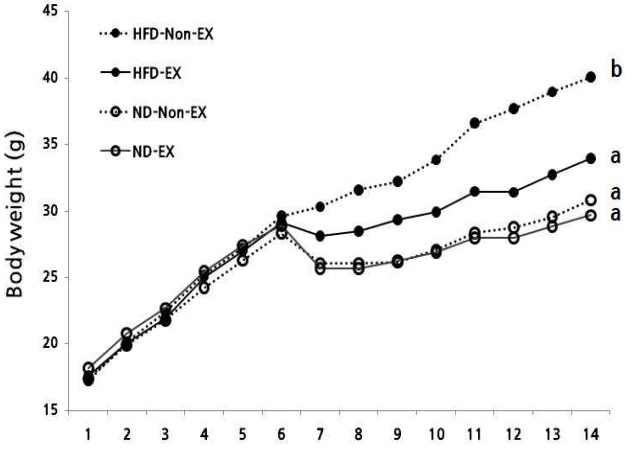

Fig. 1. Comparison of body weight among groups in intervention period for 8 weeks after obesity induced. Values with different letters are significantly different from each other ($p < 0.05$)

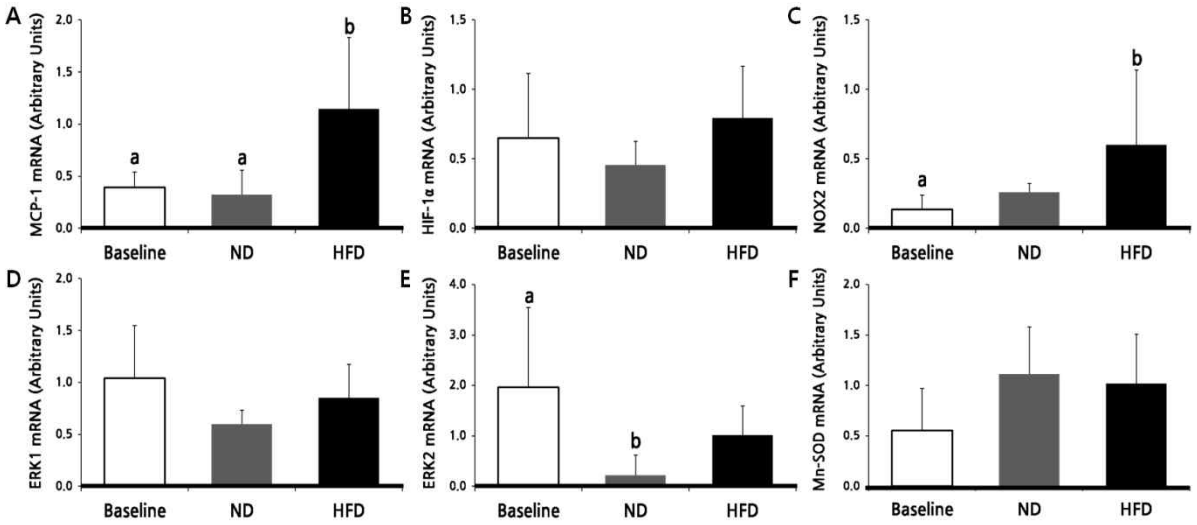

Fig. 2. Difference of MCP-1, HIF-1α, NOX2, ERK1, ERK2 and Mn-SOD mRNA expression among groups in obesity-induced periods. ND, normal diet; HFD, high fat diet. Values with different letters are significantly different from each other ($p < 0.05$)

< 0.05) than both the Baseline and ND groups (Fig. 2A). In the expression of HIF-1α mRNA, the same HFD group did not show a significant difference from the other groups; however, the group showed a somewhat little higher trend in the expression than the ND group (Fig. 2B). In the expression of NOX2 mRNA, there was no significant difference between the ND group and the Baseline group. The same HFD group, however, showed a significantly higher expression ($p < 0.05$) than the Baseline group, and a slightly higher trend in the expression than the ND group (Fig. 2C). In the expression of ERK1 mRNA, the same HFD group showed another slightly higher trend in the expression than the ND group (Fig. 2D). In the expression of ERK2 mRNA, the ND group was significantly lower ($p < 0.05$) than the Baseline group. The same HFD group showed no significant difference but a rather higher trend in the expression than the ND group (Fig. 2E). In the expression of Mn-SOD mRNA, the ND group showed a fairly higher trend in the expression than the Baseline group, and the HFD group showed a somewhat decreasing trend in the expression compared to the ND group. However, the difference among the three groups was not significant (Fig. 2F).

The effects of exercise and diet composition on mRNA expression in obese adipose tissue

Exercise and diet treatment of eight weeks was carried out after obesity was induced with a high-fat diet for five weeks. Then, the difference of mRNA expression among the groups was analyzed. The expression of MCP-1 mRNA showed significant interaction with exercise and diet ($F = 8.784$, $p < 0.05$). Although the main effect of exercise was not shown, there was diet main effect ($F = 22.376$, $p < 0.01$). The post hoc test revealed that the HFD-EX, ND-Non-EX, and ND-EX groups showed a significantly lower expression of MCP-1 ($p < 0.05$) than the HFD-Non-EX group, and there was no difference among the HFD-EX, ND-Non-EX and ND-EX groups (Fig. 3A). In the expression level of HIF-1α mRNA, exercise and diet showed significant interaction ($F = 44.489$, $p < 0.001$) with exercise ($F = 26.714$, $p < 0.001$) and diet ($F = 39.687$, $p < 0.001$) main effects. The post hoc test showed that the HFD-EX, ND-Non-EX and ND-EX groups have a significantly lower expression of HIF-1α ($p < 0.05$) than the HFD-Non-EX group, and the ND-Non-EX group had a significantly lower expression of HIF-1α ($p < 0.05$) than the HFD-EX group (Fig. 3B). In the expression level of NOX2 mRNA, exercise and diet showed a significant interaction ($F = 4.988$, $p < 0.05$) with a diet main effect but no exercise effect ($F = 15.886$, $p < 0.01$). The post hoc analysis showed the HFD-EX, ND-Non-EX and ND-EX groups had a significantly lower expression of NOX2 ($p < 0.05$) than the HFD-Non-EX group (Fig. 3C).

No interaction between exercise and diet was found in the expression level of ERK1 mRNA, however, significant main effects of exercise ($F = 8.843$, $p < 0.05$) and diet ($F = 40.213$, $p < 0.001$) were present. The post hoc test revealed the HFD-EX group showed a slightly lower trend in the expression of ERK1 than the HFD-Non-EX group, and the ND-Non-EX and ND-EX groups had a significantly lower expression ($p < 0.05$) than the HFD-Non- EX group (Fig. 3D).

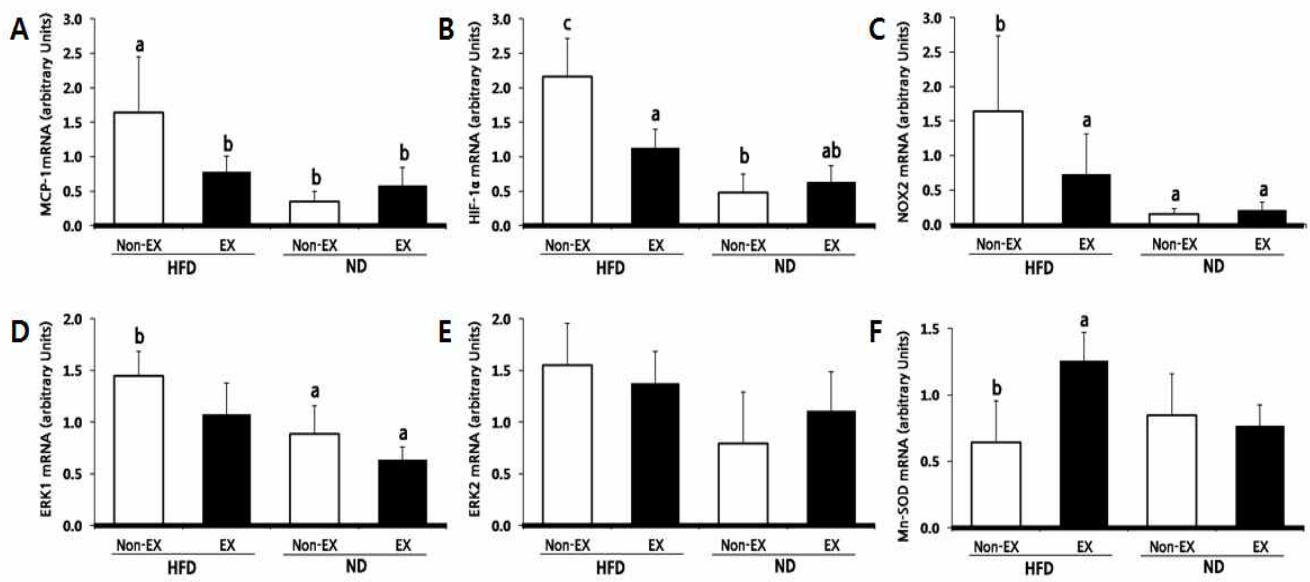

Fig. 3. Difference of MCP-1, HIF-1α, NOX2, ERK1, ERK2 and Mn-SOD mRNA expression among groups. HFD, high fat diet; ND, normal diet; HFD-Non-EX, high fat diet+non treadmill exercise; HFD-EX, high fat diet+treadmill exercise; ND-Non-EX, normal diet+non treadmill exercise; ND-EX, normal diet+exercise. Values with different letters are significantly different from each other ($p < 0.05$).

Interaction between exercise and diet was not shown in the expression level of ERK2 mRNA. However, significant main effects of diet ($F = 17.436$, $p < 0.01$), not exercise, were present. The post hoc test showed no difference among groups (Fig. 3E). In the expression level of Mn-SOD mRNA, there was an interaction between exercise and diet ($F = 12.654$, $p < 0.001$). Again, significant main effects of exercise ($F = 7.764$, $p < 0.05$), not diet, were present. Post-hoc tests showed the HFD-EX group had a significantly higher Mn-SOD expression level ($p < 0.05$) than the HFD-ND-EX group (Fig. 3F).

DISCUSSION

It is known that excessive food intake and a high-fat diet influence metabolic syndrome accompanying insulin resistance as well as increased body fat. In this study, mice with a high-fat diet for five weeks had a significant weight increase compared to mice with a normal diet. Vieira *et al*. [17] reported that a group given a 45% high-fat diet had a significant increase in weight and triglycerides concentration in the liver four weeks after the beginning of testing compared to a group with a normal diet. The same subjects exhibited a somewhat higher trend in insulin resistance than the normal diet group. The degree of obesity has static correlation with the expression of MCP-1 in adipose tissue [3,4,18], and in this study, despite the no difference in weight between HFD and ND group, high-fat diet of five weeks significantly increased the expression of MCP-1 mRNA in WAT. In the MCP-1 mRNA increased tissue, it can be estimated that the accumulation of macrophage increased due to the pivotal rol of MCP-1 in mobilizing macrophage into the obese adipose tissue. It was evident that a high-fat diet with exercise treatment, a normal diet, and a normal diet with exercise treatment for eight weeks can reduce the expression of MCP-1 in obese adipose tissue. These results suggest that regular exercise and change in diet composition can reduce the accumulation of macrophage influencing the expression of inflammatory genes in visceral adipose tissue (VAT). Also, mice with hepatic steatosis showed more inflammation in VAT than obese mice with normal amount of lipids in their livers [19]. It can be supposed from the results that a high-fat diet as a cause of inflammation can independently show abnormality of metabolism in obesity. This study shows that mice treated by both a high-fat diet and exercise treatment have a significant decrease in MCP-1 mRNA. Mn-SOD increased in VAT has a high possibility of reducing MCP-1 expression by ROS reduction rather than weight loss. A possible reason why mice treated with both a normal diet and exercise do not have a significant increase of Mn-SOD is that Mn-SOD mRNA demand may not be increased by exercise since oxidative stress in WAT is not increased by weight loss at the time of sampling.

HIF-1α is the most important signal medium for hypoxia [20] and can be used for its analysis [20,21]. The results of this study showed that mice fed with a high-fat diet for 13

weeks showed a significant increase in HIF-1α mRNA compared to the group with a normal diet after obesity was induced. A long term high-fat diet, therefore, can cause hypoxia in WAT. Recently, other studies reported the same results in obese animal models by ob/ob and KKAy and high-fat diets in mice [6-8]. Hypoxia in the adipose tissue of obese people is a high risk factor in the death of fat cells and causes macrophage to infiltrate into the adipose tissue [22]. Also, in this study, hypoxia of obese adipose tissue induced by a high-fat diet influenced the increase of MCP-1, a strong chemotactic factor of macrophage. Hypoxia, therefore, is believed to be the main cause for the accumulation of macrophage in adipose tissue. The fact that weight loss through caloric restriction reduces HIF-1α [5,8] means that the condition of oxygen in VAT is improved by weight loss. From the results of this study, it can be concluded that long term and regular exercise and change in diet composition can improve the condition of oxygen in VAT. Both exercise and fasting increase blood flow in adipose tissue and can improve insulin sensitivity [23,24]. Also, insulin resistance of endothelial cells can reduce blood flow due to malfunction [25,26]. Thus, exercise and change in diet composition may increase blood flow by improving insulin sensitivity of endothelial cells in adipose tissue and increase the possibility of improving the condition of oxygen in the tissue. Mice fed with a normal diet for only eight weeks after obesity induction of five weeks show a significant decrease in HIF-1α compared to the group treated by both a high-fat diet and exercise. This is because weight loss has more effects on HIF-1α. In the group with a high-fat diet and exercise, the antioxidant effects of exercise, such as Mn-SOD increase, might have influenced the expression of HIF-1α, as well as promoted weight loss.

NOX2 mRNA significantly increased due to a high-fat diet for five weeks according to weight gain trend. It can by hypothesized that weight gain by a high-fat diet increased oxidative stress in tissue. Recently, NADPH oxidase was proposed to act as an oxygen sensor in the reaction of hypoxia in alveolus [27]. It was shown that ROS produced by NADPH oxidase in hepatic cells of humans (HepG2 cell) is needed for activating HIF-1 [28]. In this study, NOX2 and HIF-1α mRNA were significantly increased due to a high-fat diet. This means that ROS by NADPH oxidase influenced the expression of HIF-1α mRNA. Previous research on astrocytes reported a HIF-1 bonding in the promoter of MCP-1, and that the expression of HIF-1α and MCP-1 increased due to the reaction to hypoxia [29]. HIF-1α can, therefore, be crucial in the expression mechanism of MCP-1. Also in this study, the level of MCP-1 mRNA increase by hypoxia in obese adipose tissue may be caused by signal paths of ROS and in HIF-1α by NADPH oxidase. However, since earlier research clearly explaining this mechanism in adipose tissue is insufficient, further studies are needed. In this study, mice treated by a high-fat diet and exercise, a normal diet only, or both a normal diet and exercise for eight weeks had a significant decrease in NOX2 mRNA compared to the group with only a high-fat diet. This is considered as weight loss by diet therapy. However, Sakurai, *et al*. [30] reported that exercise training of rats for nine weeks significantly decreased NOX2 in epididymal WAT through antioxidant enzymes, such as MN-SOD. Also in this study, mice treated by both a high-fat diet and exercise showed a significant increase in the expression of Mn-SOD and a decrease in the expression of NOX2 compared to mice treated by only a high-fat diet. Therefore, it was assumed that antioxidant effects reduced the expression of NOX2. It was not clear, however, whether NOX2 reduction reported by Sakurai, *et al*. [30] was attributed to the effects of weight loss or due to the antioxidant effects. Based on the results of the present study, however, it can be concluded that the reduction of NOX2 expression was caused by the independent antioxidant effects of exercise. As described above, a long term high-fat diet can cause oxidative stress in adipose tissue regardless of weight so that reduction of NOX2 expression in mice treated by both a high-fat diet and exercise is more likely to be induced by the antioxidant effects of exercise.

ERK1/2 has a sensitive role in oxidation-reduction reaction [31]. Lo *et al*. [32] reported that the expression of MCP-1 influenced the inflow of smooth muscle cells by ROS and ERK1/2. On the other hand, Sutton *et al*. [33] reported that ERK1/2 plays an important role for the expression of HIF-1α as a reaction to hypoxia, but it is not necessary. In analyzing epididymal adipose tissue, the current study does not support previous research. However, EPK1 and 2 showed an increasing trend in all groups except the Baseline group as the weight of the mice increased, and the expression of EPK1 presented a decreasing trend as weight of the mice decreased. So the possibility that EPK1 and 2 may influence the expression of HIF-1α and MCP-1 mRNA by hypoxia in adipose tissue and ROS can be proposed. Future research can further elucidate this result.

In conclusion, NADPH oxidase by hypoxia and HIF-1α by hypoxia can influence the expression of MCP-1 in VAT. Long term and regular exercise can reduce the expression of NOX2, HIF-1α and MCP-1 affecting inflammation induction in VAT and the accumulation of macrophage caused by obesity or a high-fat diet. The reduction of NOX2, HIF-1α and MCP-1 expression can be accomplished by weight loss through exercise and change in diet composition, or through antioxi-

dant effects of exercise, such as Mn-SOD. Moreover, despite continued high fat diet, it was verified that the antioxidant effects and improvement of hypoxia through exercise can significantly reduce the expression of MCP-1 in VAT.

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