

Combined influence of dietary restriction and treadmill running on MCP-1 and the expression of oxidative stress-related mRNA in the adipose tissue in obese mice

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Nayoung Ahn and Kijin Kim. Combined influence of dietary restriction and treadmill running on MCP-1 and the expression of oxidative stress-related mRNA in the adipose tissue in obese mice. *JENB*, Vol. 18, No. 3, pp.311-318, 2014 **[Purpose]** This study suggests that the negative effects of inflammation caused by obesity could be prevented through diet restriction and exercise. **[Methods]** In this study, 44 C57/BL6 male mice at about 4 weeks old (Orient bio, South Korea) were given a high fat diet for 5 weeks to make them obese. To help the mice lose weight, their dietary intake was limited and they were exercised on the treadmill for 8 weeks, and during that period, we analyzed the changes of MCP-1, ERK, Mn-SOD, HIF-1, and NOX in epididymal adipose tissue. There ND control group and obese group with high fat diet (HFD), and it is divided into four groups; HFD-ND-EX group, HFD-ND-nonEX group, HFD-DR-EX group and HFD-DR-nonEX group. **[Results]** During their progress, the mRNA expressions of HIF-1 α and ERK2 decreased, as did the expression of MCP-1 contained in the nucleus by suppressing oxygen free radicals, which was observed after the exercise program. However, dietary restriction without exercise training triggered an increase in the mRNA expression of MCP-1. **[Conclusion]** To put this in perspective, combining exercise and dietary intake restriction likely prevented an influx of macrophages by reducing the number of fat cells, whereas only dietary restriction was not effective against reducing inflammation. **[Key word]** obesity, exercise, diet restriction, MCP-1, inflammation.

INTRODUCTION

Inflammatory cytokines and chemokines produced and secreted by fat tissue due to excessive accumulation and the enlargement of adipose tissue are considered to be the most likely cause of chronic inflammation that is believed to play a role in several metabolic diseases [1]. And the failure of some hormones with structurally the same signaling pathway as cytokines to secrete can reduce insulin sensitivity, which may lead to the development of type 2 diabetes, cardiovascular diseases, and atherosclerosis [2].

Cytokines are classified as IL (interleukins), TNF (tumor necrosis factors), IFN (interferons), CSF (colony stimulating factors), TGF (transforming growth factors), and chemokines, and form the intracellular signaling network. Chemokines, in particular, play a key role in inflammatory cell mobilization during the development stages of an inflammatory disease

[3,4]. Furthermore, MCP-1 (monocyte chemoattractant protein-1) is the most important chemokine among them that can control the transfer of monocytes and is widely spread in atherosclerotic lesions, including vascular endothelial cells, smooth muscle cells, monocytes, and macrophage [5,6].

Recent studies have shown that the expression of MCP-1 is much more marked in mice fed a high fat diet, and similarly associated chemokines interact with each other [2]. The expression of MCP-1 is affected by the production of reactive oxygen species, which include signaling molecules such as ERK (extra cellular signal regulate kinase), Mn-SOD (manganese containing superoxide dismutase), HIF-1 (hypoxia inducible factor), and NOX (nitric oxide synthase). NOX2, in particular, is located in the cell plasma membrane, and combines with membrane protein (p22phox) and cytoplasmic protein (p47phox, p67phox and p40phox) to activate NADPH oxidase. Accordingly, it is expected to provide an important target to

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determine an effective way to prevent obesity. By analyzing how hypoxia inducible factor (HIF)-1 α and NOX2, and the member of NADPH oxidases that are specifically expressed in white adipose tissues under hypoxic condition affect the expression of MCP-1 gene in adipose tissue, and then observe the change that is displayed after dietary changes and exercise, we can develop an effective method to prevent obesity.

However, analysis of the anti-oxidative effect that occurs in the body by exercising-responding to the factors that affect the infiltration of macrophages and inflammation in adipose tissue, is lacking. The types of cytokine genes expressed that cause inflammation in obesity will appear different depending on diet composition or exercise types. Also, the effect of excessive dietary restriction on the production of oxygen free radicals and oxidative damage to DNA has not been verified with certainty yet.

Therefore, in this study, the combined therapies of dietary restriction and an exercise program for HFD obese mice were done to determine what kind of changes in the expression of MCP-1, ERK, Mn-SOD, HIF-1, and NOX in epididymal adipose tissue occurred in order to examine the possibility that the combined therapies of dietary restriction and exercise as a method to prevent and treat negative effects of inflammatory disease.

METHODS

Subjects

Subjects of the study were 44 C57/BL6 male mice at about four weeks old. All mice were acclimated to the environment through a preliminary breeding program for 1 week, where they were fed a high fat diet for 5 weeks to make them obese. After that, their dietary intake was limited and they were exercised on the treadmill to treat obesity for 8 weeks. Names of each group are the same as shown on <Table 1>.

Table 1. Treatment groups

Groups	Classification criteria
ND-ND	After preliminary breeding for 1 week, normal diet.
HFD-ND-Non-EX	After obesity induced by high fat diet for 5 weeks, normal diet with no exercise.
HFD-ND-EX	After obesity induced by high fat diet for 5 weeks, normal diet with exercise for 8 weeks.
HFD-DR-Non-EX	After obesity induced, dietary restriction and no exercise.
HFD-DR-EX	After obesity induced, dietary restriction and with exercise for 8 weeks.

ND: normal diet, HFD: high fat diet, DR: dietary restriction EX: exercise

Table 2. Number of subjects

Group	ND-ND	HFD-ND		HFD-DR	
		Non-EX	EX	Non-EX	EX
Number	8	8	7	10	11

ND: normal diet, HFD: high fat diet, DR: dietary restriction EX: exercise

Table 3. Component of high fat diet and normal diet

Products	High-fat Diet (D12451)		Normal Diet (D12450B)	
	gm%	kcal%	gm%	kcal%
Protein	24	20	19.2	20
Carbohydrate	41	35	67.3	70
Fat	24	45	4.3	10
Total		100		100
kcal/mg	4.73		3.85	

Experimental procedures

Induction of obesity

C57/BL6 mice were induced obesity for 5 weeks, the obesity treatment group was composed of 12 mice each. However, there are differences in the number of mice in each group as shown in <Table 2> because some of them were sacrificed during the course of the study. Mice on a high fat diet (D12451, Research, Inc.) were given ad lib access to food contained 45% fat and drinking water. Mice with normal diet were given ad lib access to a diet that contains 10% fat and water. The details are as shown on <Table 3>. During the breeding period, light-dark cycle of the day was set to 12 hours. Temperature and relative humidity of the breeding room is maintained around $24 \pm 1^\circ\text{C}$ and 60% each with 4 mice per cage. During the breeding process, mice were the same controlled environment and the daily food intake and body weight were measured daily.

Treatment program

Diet restriction

In the HFD-ND and HFD-ND-EX groups, mice were given ad lib access to a normal diet contains 10% fat, and their daily intake was calculated. The HFD-DR group was given ad lib access to a normal diet that contained 10% fat only about 65% (about 2.0 g/mouse) by applying the intake restriction method, compared to that of the average daily intake (about 3.0 g/mouse) of induction of obesity period. Intake is the weight of the diet given to them for the day minus the weight of diet measured after 24 hours, and was measured every day.

Exercise program

Targeting HFD-ND-EX and HFD-DR-EX group, treadmill exercise was applied. To get the mice adapted to the treadmill

mice were put on it 30 minutes a day, 3 times a week, at a rate of 10-18m/min for one week. After that, treadmill running for was conducted, 35-64 minutes a day, 5 days a week for 8 weeks to reduce obesity. The treadmill's speed and incline was adjusted to allow the mice to reach 60-75% of their maximum heart rate, a modified method from a previous study [7].

Combined programs of dietary restriction and exercise

Only the HFD-DR-EX group combined dietary restriction and exercise. The dietary restriction applied was 2.5 g per mouse (10 g per a cage). Exercise training is applied in the same manner as HFD-ND-EX group, but was conducted for 8 weeks and the frequency of exercise was reduced to three days a week.

Measurement items and analytical methods

The body weight of mice was measured at weekly intervals over the whole experiment time using Dial-O-Gram® Balance (OHAUS, USA). The degree of expression of HIF-1 α , NOX2, ERK1/2, and MCP-1 in epididymal adipose tissue and mRNA expression of Mn-SOD was analyzed after obesity treatment. The mice were sacrificed to eliminate the effect of the one-off exercise 24 hours after the last treadmill session. In this method of sacrifice, after being systemically anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight), the mice were freeze-clamped at -70°C in liquid nitrogen and stored until needed for analysis.

Total RNA extraction

Total RNA was extracted from epididymal adipose tissue by the following method. First, TRI reagent (Sigma-Aldrich, Inc., USA) was added in 50-100 mg aliquots to the tissue, which was ground for 30 seconds to 1 minute on ice using a homogenizer (PRO Scientific Inc, U.S.A). Chloroform (200 μ l) was added to the pulverized tissue and mixed well with a vortex mixer and allowed to stand for 10 minutes at room temperature. The supernatant was separated by centrifugation at 13,000 rpm for 15 minutes in a temperature of 4°C using a centrifugal separator and aliquoted out into another tube (centrifuge 5415 R, Eppendorf, Germany). Isopropanol (500 μ l) was added to the supernatant and centrifuged at 13,000 rpm for 10 minutes at a temperature of 4°C, and everything was removed but pellet. The remaining RNA pellet was washed with 70% alcohol and dissolved in a water bath at 55-60°C for 15 minutes with the addition of DEPC-treated (diethyl pyrocarbonate) deionized water. The concentration needs to be calculated by measuring the absorbance at 260 nm using a UV spectrophotometer (UV-mini1240, Shimadzu Co., Japan). After that, DNA and DNase that may be conta-

minating the RNA extracted was removed using the TURBO DNA-free™ Kit (Ambion Inc., USA).

cDNA synthesis

cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). For the synthesis, 5 \times iScript reaction mix (4 μ l) and iScript reverse transcriptase (1 μ l) was added to the RNA (1 μ g) extracted from each sample. Nuclease-free water was added to make the total amount 20 μ l. It was cultured continuously for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C by using the ASTEC PCR Thermal Cycler (PC320; ASTEC, Japan).

Real-Time PCR

Real-time PCR used synthesized cDNA as a template, and each specific primer of HIF-1 α (Forward 5'-GCACTAGACA AAGTTCACCTGAGA-3', Reverse 5'-CGCTATCCACATC AAAGCAA-3'), NOX2 (Forward 5'-TTGGGTCAGCACTGG CTCTG-3', Reverse 5'-TGGCGGTGTGCAGTGCTATC-3'), ERK1 (Forward 5'-CGGCTGAAGGAGTTAATCTT-3', Reverse 5'-GAAGGAGACAGGTAGGAGCAG-3'), ERK2 (Forward 5'-ACCGTGACCTCAAGCCTTC-3', Reverse 5'-TGATCTG GATCTGCAACACG-3'), MCP-1 (Forward 5'-ACTGAAGC CAGCTCTCTCTTCCTC-3', Reverse 5'-TTCCTTCTTGGGG TCAGCACAGAC-3'), Mn-SOD (Forward 5'-CATTAACGC GCAGATCATGC-3', Reverse 5'-CCCAAAGTCACGCTTG ATAGC-3') and GAPDH (Forward 5'-TGTGGATCTGACAT GCCGC-3', Reverse 5'-GAATGGGAGTTGCTGTTGAAG-3') were mixed with the enzyme reagent reaction 2X SYBR Green Supermix (Bio-Rad, USA), and were amplified in the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). After the PCR reaction, the degree of expression of the mRNA of HIF-1 α , NOX2, ERK1, ERK2, MCP-1 and Mn-SOD, was quantified as the ratio of the GAPDH house-keeping gene.

Statistical analysis

The average and the standard deviation of each group by using SPSS 17.0 statistical program was calculated for all data. One-way ANOVA was used to determine the difference in the average of the baseline variables between groups. If a significant difference between the groups is observed, the post-hoc test was conducted by the Tukey method. A Paired t-test was applied to differences in the measured variables of HFD-ND-DR group and HFD-ND group, and the difference between in the presence or absence of the exercise was analyzed by an Independent t-test. The level of statistical significance was set to a p-value of less than 5% ($p < .05$).

RESULTS

The change due to dietary restriction after induction of obesity

Changes in each factor corresponding to HFD-ND and HFD-ND-DR were conducted for 8 weeks after induction of obesity for 5 weeks are the same as shown on <Table 4>. Body weight of HFD-ND-DR group was significantly ($p < .001$) lower compared to ND-ND and HFD-ND groups. However, the expression of MCP-1 mRNA was significantly ($p < .001$) higher in HFD-ND-DR group compared to the other two groups. NOX2 was significantly ($p < .05$) lower in HFD-ND-DR group than in the ND-ND group, and ERK2 was significantly ($p < .05$) lower in HFD-ND group than ND-ND group. No significant differences in the expression of HIF-1 α , Mn-SOD and ERK1 were observed between exercise and no exercise groups.

Table 4. Differences in mRNA expression of inflammatory markers due to diet restriction after diet-induced obesity.

Item	ND-ND	HFD-ND	HFD-DR	F-value	Post-hoc
Body Weight (g)	30.41 \pm 2.31	30.83 \pm 3.17	22.46 \pm 2.43	29.387***	a,b > c
MCP-1	0.48 \pm 0.23	0.35 \pm 0.15	3.50 \pm 3.22	7.212**	a,b < c
HIF-1 α	0.77 \pm 0.23	0.48 \pm 0.28	0.68 \pm 0.48	1.457	
NOX2	0.36 \pm 0.13	0.15 \pm 0.08	0.14 \pm 0.25	4.112*	a > c
Mn-SOD	0.72 \pm 0.28	0.84 \pm 0.31	0.70 \pm 0.27	0.643	
ERK1	0.95 \pm 0.14	0.89 \pm 0.27	0.95 \pm 0.23	0.240	
ERK2	1.48 \pm 0.49	0.80 \pm 0.50	1.14 \pm 0.46	4.102*	a > b

Values are mean \pm SD

a: ND-ND, b: HFD-ND, c: HFD-DR, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The change corresponding to the combination of dietary restriction and exercise after induction of obesity

Changes in each factor corresponding to the combination of dietary restriction and exercise after induction of obesity are the same as shown on <Table 5>. Body weight of HFD-DR group was significantly reduced in Non-Ex ($p < .001$) and Ex groups ($p < .05$) when compared with HFD-ND group. The expression of MCP-1 mRNA was significantly ($p < .01$) higher in Non-ex groups than the Ex groups in HFD-DR group in particular. The expression of HIF-1 α mRNA was significantly ($p < .05$) lower in Ex groups than

Table 5. Changes in mRNA expression of inflammatory markers with or without exercise

Item		HFD-ND	HFD-DR
Body Weight (g)	Non-EX	30.83 \pm 3.17	22.46 \pm 2.43####
	EX	29.69 \pm 2.70	27.31 \pm 1.75***#
MCP-1	Non-EX	0.35 \pm 0.15	3.50 \pm 3.22#
	EX	0.58 \pm 0.26	0.06 \pm 0.05####
HIF-1 α	Non-EX	0.48 \pm 0.28	0.68 \pm 0.48#
	EX	0.64 \pm 0.23	0.31 \pm 0.29*#
NOX2	Non-EX	0.15 \pm 0.08	0.14 \pm 0.25##
	EX	0.21 \pm 0.12	0.21 \pm 0.15
Mn-SOD	Non-EX	0.84 \pm 0.31	0.70 \pm 0.27
	EX	0.77 \pm 0.16	0.67 \pm 0.56
ERK1	Non-EX	0.89 \pm 0.27	0.95 \pm 0.23
	EX	0.64 \pm 0.12*	0.76 \pm 0.38
ERK2	Non-EX	0.80 \pm 0.49	1.14 \pm 0.46#
	EX	1.11 \pm 0.37	0.53 \pm 0.47**#

Values are means \pm SD

$p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ Compared between HFD-ND and HFD-DR

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Compared between Non-Ex and Ex

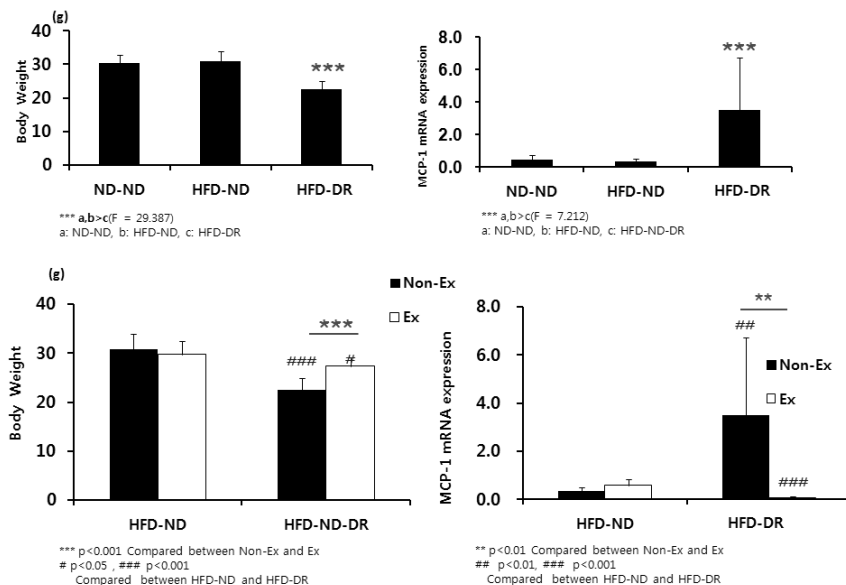


Fig. 1. Changes in mRNA expression of inflammatory markers due to exercise and dietary restriction.

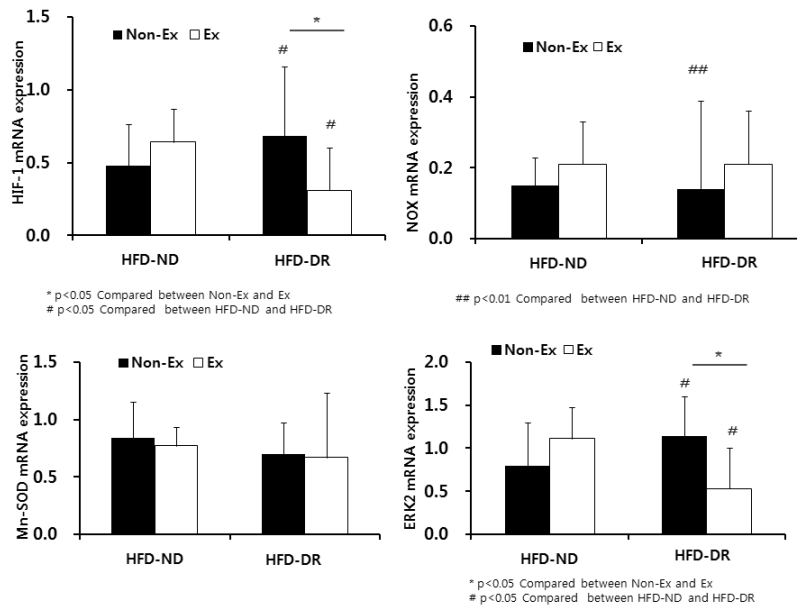


Fig. 2. Changes in chemokines due to with or without the exercise and dietary restriction.

in the Non-Ex groups. In the Non-Ex group, the expression of NOX mRNA was significantly ($p < .01$) lower in HFD-DR group compared to the HFD-ND group. The expression of ERK2 mRNA was significantly ($p < .05$) lower in Ex groups than in HFD-ND-DR group. No significant difference was observed in the mRNA expression of Mn-SOD and ERK1 between the Ex and Non-Ex groups.

DISCUSSION

The increase in size and the number of adipose cells by obesity increases the oxidative stress in the tissue, increasing the expression of various adipokines that induce inflammation by acting on adipose tissue. Adipose tissue plays a critical role in various inflammatory reactions including the stromal vascular fraction within adipose cells, as well as blood cells, endothelial cells, and macrophages [8-10]. In obesity, there is an increase in the size and reduction in blood flow of adipocytes. Adipocytes larger than 150-200 μm in diameter lose the interval that allows for the normal diffusion of oxygen, resulting in oxygen deficiency and hypoxia [11]. This oxygen-deficient state of white adipose tissue is associated with the increased production of reactive oxygen species [12]. Nitric oxide synthase corresponding to the major source of intracellular reactive oxygen species and oxidation-induced enzymes such as NADPH oxidase acts to induce the expression of various adipokines including MCP-1 and adiponectin is markedly increased in adipose tissue of the

obese [13].

Macrophages entering white adipose tissue induces an inflammatory state, and the molecular mechanism by which it enters into the adipose tissue of the obese is not understood. Macrophages optionally exert function by each of the cytokines cause the polarization [14]. M1 macrophages induce the expression of classic activation signals IFN- γ , LPS, and TNF- α , and cause a Type-1 inflammatory action because of the increase of their increased iNOS (NOS2) levels. Meanwhile, M2 macrophages alternative to pro-inflammatory action and anti-inflammatory action [15]. The pro-inflammatory cytokines that is produced in adipose tissue in M1 macrophage are MCP-1, TNF- α , and IL-6 [16]. MCP-1 is part of the CC-chemokine system that promotes the migration of inflammatory cells into tissue by chemotaxis and cell adhesion via integrin activation.

Levels of MCP-1 expression in adipose tissue and plasma MCP-1 concentrations were found to be a positively correlated with the severity of obesity [9,10,17] and with markers of macrophages in adipose tissue [18]. MCP-1 is expressed before other markers in adipose tissue macrophages due to obesity [10], which plays a key role in macrophages entering into adipose tissue [19].

In this study, the expression of MCP-1 mRNA in adipose tissue was significantly ($p < .01$) higher in Non-ex group than than in the HFD-DR group that was exercised, and the expression of MCP-1 mRNA was significantly ($p < .05$) higher in the HFD-ND-DR group than in the HFD-ND group in Non-Ex groups. However, the expression of MCP-1 mRNA

and weight loss were significantly ($p < .01$) lower in HFD-DR group that was exercised compared to the HFD-ND group that was exercised. Therefore, we conclude that the expression of MCP-1 mRNA is a pro-inflammatory factor that can be reduced by exercise and dietary restriction. Dietary restriction for obesity treatment slows aging and prevents chronic diseases caused by reducing inflammation [20]. Reducing inflammation by dietary restriction has a positive effect specific on metabolism and the expression of hormone and gene. It cannot be ruled out that excessive dietary restriction may induce the expression of inflammation through other metabolic mechanisms and altering hormones.

The HIF-1 α is the most important signal of oxygen deficiency in the body [21], its expression level increased [18] in the adipose tissue of obese patients. HIF-1 α protein levels of the oxygen-deficient state rapidly increases through the inhibition of degradation by HIF-1 α ubiquitination-proteasome parameters. In this study, HIF-1 α mRNA was significantly ($p < .05$) higher in the DR-Non-EX group as compared to all of the other groups. Therefore, we conclude that exercise plays a critical role in reducing oxygen deficiency in the body, and in addition, reduces the expression of HIF-1 α mRNA from adipose cells and suppresses the expression of inflammation.

NADPH oxidase of the NOX family is an important source of reactive oxygen species in a variety of cells [22,23]. NOX2 (gp91phox) is located in the plasma membrane of cells, and binds to a membrane protein (p22phox) and cytoplasmic proteins (p47phox, p67phox and p40phox) to activate NOX2 (22). The NADPH oxidase complex is related to an increase in peroxides and lipid peroxides of adipose tissue by obesity and increased production of reactive oxygen ions (O₂⁻) [13,24], and induces oxidative stress and adipokine dysregulation in adipose tissue [13]. In this study, there was no significant difference in NOX2 mRNA expression between the exercise or no exercise groups. However, when the comparison was based on dietary intake, the expression in the DR group was significantly ($p < .01$) lower, demonstrating the effect of weight loss (Table 5) through dietary restriction on NOX2 mRNA expression level in the adipose tissue.

Reactive oxygen species play a role in intracellular signaling, and in order to transmit the nuclear signal, they have a sensitive site that reacts to redox reactions that transmits a signal to the nucleus to induce the expression of the mitogen-activated protein kinase (MAPK) ERK (Extra cellular signal regulate kinase). Thus, MAPKs are able to respond to ROS. Of all the MAPKs, ERK has been reported to be associated with expression of the MCP-1 gene in different cell types. In this study, ERK2 mRNA was

significantly ($p < .05$) lower in DR-EX group, and thereby, we concluded that combining exercise and dietary intake restriction are effective in reducing the onset of inflammation. Therefore, there is a possibility that ERK is performing the role of an important mediator in the gene expression of MCP-1 by oxidative stress in the adipocytes of obese patient, and it is likely that ERK2, one of ERK1 and ERK2 of 2 subtypes of ERK, affects the gene expression of MCP-1.

Long-term regular exercise was found to reduce on oxidative stress in adipose tissue after diet-induced obesity with a high fat diet, due to an increase in intrinsic antioxidant enzymes such as manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPX) [25]. In addition, the effect of weight loss due to the combination of dietary restriction and exercise reduce the influx of macrophages in adipose tissue through the inactivation of HIF-1 α and ERK2, decreasing the expression of pro-inflammatory factors. However, further studies will provide a basis for presenting more effective obesity treatment methods by performing a detailed analysis such as a reduction in the size of adipose tissue, changes in exercise capacity, and a variety of macrophages or cytokines polarization when applied separately or concurrently with exercise and dietary restriction.

CONCLUSION

Exercise and dietary restriction in diet-induced obese mice for 8 weeks reduced the expression of HIF-1 α , and ERK2, and MCP-1 mRNA. However, it is restricting dietary intake without exercise may significantly increase the expression of MCP-1 mRNA.

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