Original Article

The effect of aerobic exercise training on gene expression of beta3-adrenergic receptor and beta-arrestin2 in inguinal white adipose tissue of mice fed with a high fat diet

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Abstract

Background and aims: Beta-adrenergic signaling deficiency has been established to be related to obesity and related diseases. Beta3-adrenergic receptor (Adrb3) and beta-arrestin2 (Barr2) are pivotal agents in the beta-adrenergic-signaling pathway. This study aimed to investigate the preventive effect of aerobic training on dysregulation of Adrb3 and Barr2 gene expression that was induced by high-fat diet (HFD) in inguinal white adipose tissue of mice.
Materials and Methods: Twenty-one C57BL/6 mice were assigned to three groups as follows: 1) control group (n=7), 2) high-fat diet-induced overweight (HFD-OW) (n=7), and 3) high-fat diet with aerobic training (HFD-AT) (n=7). The HFD-OW group were fed with a HFD for 12 weeks. The HFD-AT group had aerobic training for six weeks on a treadmill in addition to feeding with the HFD. The real-time polymerase chain reaction (PCR) method was used to measure the gene expression of Adrb3 and Barr2 in inguinal white adipose tissue.
Results: The gene expression of Adrb3 did not significantly change between groups ($P>0.05$). However, the expression of Barr2 in HFD-OW group was significantly increased as compared to the control group (1.5-fold: $P=0.001$). Interestingly, the Barr2 expression in HFD-AT group was significantly lower compared with HFD-OW group ($P=0.045$).
Conclusion: The results indicated that aerobic training could inhibit the upregulation of Barr2 induced by HFD. It seems that a portion of the preventive effect of aerobic training on the development of obesity may be mediated by inhibiting the Barr2 expression in adipose tissue.
Keywords: Obesity, High-fat diet, β-Arrestin, Exercise training, White adipose tissue

Introduction

The worldwide prevalence of overweight and obesity remarkably has increased over the current century to an extent that one-third of the world population is currently classified as obese/overweight (1). Obesity is considered an important risk factor for metabolic syndrome, type 2 diabetes mellitus, and cardiovascular diseases (2). Therefore, prevention and treatment of the risk factor are important. Many cellular mechanisms are involved in the development of obesity and related diseases (3). β-adrenergic signaling, which plays a critical role in metabolism, is well established to be closely related to obesity (4, 5). Beta3-adrenergic receptor (Adrb3) is a main agent of the β-adrenergic signaling pathway in rodent adipocytes (6). Adrb3 plays a pivotal role in the control of adipose tissue metabolism, thermogenesis (6,7). Studies have shown that the expression of Adrb3 has been downregulated in white adipose tissue (WAT) of obese

rodents (8,9) and obese/overweight humans (8,10,11). β-Arrestin (Barr) is another agent of the β-adrenergic signaling that could be complicated in obesity (12). The Barr2s constitute a small family of proteins that negatively regulate signal transduction at G protein-coupled receptors, thereby playing a physiological and pathophysiological role in different cells (13,14). It was recently shown that Barr2 can disturb β3-adrenergic signaling in adipose tissue, thereby contributing to adiposity and related complications in a pathophysiologic manner (15). In this matter, the Barr2 was shown to impair the browning of WAT, defect nonshivering thermogenesis, and predispose mice to high-fat diet (HFD)-induced obesity (15). Interestingly, Pydi et al found that Barr2 expression was upregulated in subcutaneous adipose tissue of obese individuals (15). Therefore, it seems that Barr2 may be a negative regulator in obesity. In this light, finding a medical procedure that can inhibit Barr2expression may

be efficient in preventing and treating obesity and related metabolic disorders.

Aerobic exercise training is well recognized as an effective medical procedure to prevent and treat obesity and related complications (16). It has been shown that aerobic training can affect many cellular signaling pathways in adipose tissue (17,18). Specifically, the enhancing role of aerobic training in β-Adrenergic signaling has been reported in the WAT of mice (19). Few studies also reported that Adrb3 expression has been upregulated in adipose tissue following aerobic training in obese and nonobese rodents (20,21). Based on these findings, we postulated that aerobic training could also modulate another agent of β-Adrenergic signaling such as Barr2expression in HFD-induced obesity/overweight.

Therefore, the study aimed to survey the effect of aerobic training on gene expression of Adrb3and Barr2in WAT of mice that were predisposed to overweight by HFD.

Materials and Methods
Animals
Twenty-one C57BL/6 male mice (four weeks old, body weights of 11±2 g) were obtained from Experimental Study Center of Iran University of Medical Sciences and kept under controlled conditions (at 23°C±2°C with a humidity of 55%±10% and a 12-hour light/dark cycle). The mice were fed with standard rodent chow ad libitum with water. After a one-week acclimation, the mice were randomly assigned to three groups including 1) control (CON, n=7), 2) HFD-induced overweight (HFD-OW, n=7), and 3) HFD with aerobic training (HFD-AT, n=7). The room temperature in which mice were kept was increased to 26°C. The mice in HFD-OW group were fed with a HFD for 12 weeks. The mice in ET-AT group were subjected to aerobic training for 6 weeks along with feeding with HFD. After the experiment, mice were euthanized and blood and WAT samples were collected.

High-fat diet
The mice in HFD-OW and HFD-AT groups were fed with a HFD for 12 weeks from 5 to 17 weeks of age. The HFD contained 45 kcal% fat, 35% carbohydrate, and 20% protein (total calorie 4.78 kcal/g) (22). The mice in the control group were fed with a normal diet (15 kcal% fat, 60 kcal% carbohydrates, and 20 kcal% protein; total calorie 3.80 kcal/g) (23). The diets were made based on diet formula (Research Diets D12451 and D12450H) by the Research and Development Unit of Behparvar Animal Chow Company in Karaj, Iran.

Aerobic-training protocol
The mice in the ET-OW group were subjected to aerobic training for 6 weeks in addition to feeding with HFD. The 6-week training was initiated from the age of 11 weeks and terminated at the age of 17 weeks. The Aerobic training was done as a continuous running protocol on a rodent treadmill (Andisheh-Sanat, made in Iran) at 0% inclination. This protocol which was designed based on previous studies (24-26) was performed five sessions per week, for 6 weeks and the training load was progressively increased from 15 minutes per session at 14 m/min in the first week to 30 minutes at 20 m/min in the last week of the protocol. The control group did not have any programmed activity.

The animals were euthanized 48 hours after the last exercise training session to avoid exercise-related acute effects.

Body weight measurement
Bodyweight was weekly measured and recorded during the experiment using electronic digital scales (ENTRIS 3202-1S S, Artorius, Germany).

Tissue and blood collection
At the end of the experiment and after fasting overnight, the mice were euthanized by intraperitoneal injection of a mixture of xylazine (30 mg/kg) and ketamine (240 mg/kg) (27). The blood samples (0.7 mL) were obtained from a cardiac puncture using a heparinized syringe and put immediately into siliconized disposable glass. Then, plasma was separated by centrifugation (10 minutes, 3000 rpm, 4°C) (Avanti J 251; Beckman, USA) and frozen (-78°C) for future measurement. Inguinal subcutaneous fat pads were quickly dissected out and frozen in liquid nitrogen and stored at −80°C for future measurement.

Measurement of plasma parameters
Plasma glucose was measured by glucose oxidase method (GOD-PAP) using a detection kit (Pars Azmun, Iran, 132504H917) and an autoanalyzer (Hitachi 902, Boehringer Manneheim, Germany) (28). Sensitivity and intra- and inter-assay coefficient of variations were 2 mg/dL, 1.8% and 1.2%, respectively.

Triglyceride (TG) level was determined using an enzymatic-colorimetric method by commercial kit (Pars Azmun, Iran, 117504H917) and an autoanalyzer (Hitachi 902, Boehringer Manneheim, Germany) (28). Sensitivity and intra- and inter-assay coefficient of variations were 2 mg/dL, 1.7% and 1.5%, respectively.

Gene expression evaluation
RNA extraction
Approximately 50 mg of adipose tissue samples was homogenized in 1 mL of TRIzol reagent (Thermo Fisher Scientific, US). The total RNA was isolated using TRIzol in accordance with the manufacturer's instructions (Cat. No. 15596026). RNA concentration and purity were assessed (OD: 260/280) using spectrophotometers (Ultrospec 3000, Pharmacia Biotech, Sweden). The samples which had a ratio above 1.6 were selected for future procedures.
cDNA synthesis
First-strand complementary DNA (cDNA) was synthesized from 1 μg of RNA using Invitrogen SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, USA)

according to the manufacturer's instructions (Cat. No. 18-064-014).

Primers

The primers were designed based on the methods used in previous studies (15), verified using primer-blast/NCBI and Oligo Analyzer software (1.02), and synthesized by SinaClon (Iran). The characteristics of the used primers have shown in Table 1.

Real-time polymerase chain reaction

The mRNA contents of target genes were determined by real-time polymerase chain reaction (PCR) performed using the Rotor-Gene 6000 instrument (Corbett, Australia) with SYBR Green Quantitative RT-qPCR Kit (QR0100-Sigma-Aldrich, Germany) and specific primers, according to the manufacturer's instructions (Cat. No. 1907/2006). The thermal cycling profile included denaturation at 95°C for 10 minutes, followed by 36 cycles at 95°C for 10 seconds and 56°C for the 30 seconds according to the optimized annealing temperature of target primers.

Quantification of relative gene expression

The efficiency of PCR product and primers was determined by LinRegPCR software (version 2020.0), and the specificity of PCR products was confirmed by melting curve analysis. The relative changes in gene expression were quantified by Pfaffl method (29) (is shown below) and represented as fold change using Genex software (7.0). GAPDH served as the endogenous control.

$$Fold\ Change = \frac{E_{Tar.}^{\Delta cp_{Tar.}^{(Con.-treat.)}}}{E_{Ref.}^{\Delta cp_{Ref.}^{(Con.-treat.)}}}$$

Statistical analysis

Data were statistically analyzed and graphs were drawn using GraphPad Prism software version 8.0 (Irvine, CA, USA). Normal distribution of the data was verified using the Shapiro-Wilk test. Brown-Forsythe test was used to examine the homogeneity of the variances among groups. The data were reported as the mean ± standard error of mean (SEM). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's test. $P \leq 0.05$ was considered statistically significant.

Results

General characteristics

The general characteristics of the mice including variables of body weight and plasma levels of glucose and TG are shown in Table 2.

Bodyweight progressively increased over the time course of the experiment in all groups (Figure 1). After 12 weeks of the experiment, the animals in HFD-OW group displayed a significantly increased body weight compared to the control group ($P=0.001$) (Figure 1). However, the HFD-AT group did not show a significant increase ($P=0.162$) (Table 2). Analysis of weight gains (before and after the experiment) showed a significant increase in HFD-OW group (63%, $P<0.001$)) and HFD-AT group (40%, $P=0.010$) compared to the control group (Table 2). The HFD-AT group showed a (non-significantly) lower weight gain compared to HFD-OW group (14%, $P=0.176$) (Table 2).

Both HFD-OW and HFD-AT groups displayed significantly higher fasting levels of glucose and TG compared with the control group ($P<0.05$) (Table 2). These values in HFD-AT group were lower (but not significantly, $P>0.05$) when compared to HFD-OW group (Table 2).

Table 2. General characteristics of the mice in different study groups

	Control (n=7)	HFD-OW (n=7)	HFD-AT (n=7)
Body weight (g) (pre)	12.85±0.55	13.07±0.68	12.45±0.63
Body weight (g) (Post)	24.14±0.87	31.49±2.09**	28.31±1.32
Weight gain (g) (post-pre)	11.28±0.33	18.42±1.45***	15.85±0.71**
Glucose (mg/dL) (Post)	85.91±6.01	148.0±12.33**	120.45±8.45*
TG (mg/dL) (Post)	90.45±5.13	145.68±9.85***	121.66±6.43**

HFD-OW: High-fat diet-induced overweight; HFD-AT: a high-fat diet with aerobic training; Pre: before experiment; Post: after the experiment; TG: triglyceride

Data are presented as mean±SEM. Data were analyzed using one-way ANOVA followed by Tukey's test. * $P<0.05$ ** $P<0.01$ *** $P<0.001$ compared to the control group.

Table 1. Characteristics of the used primers

| Name	Accession number	Sequences 5′→3′	Tm °C	GC %	Product length (bp)	Reference
β-Arrestin1 (Arrb1)	NM_177231.2	CCAAACCTTCCATGCTCAGT TAGCCGCACAGAGTTCCTTT	58.08 59.31	50.00 50.00	138	(15)
β-Arrestin2 (Arrb2)	NM_001271358.1	AAGTCGAGCCCTAACTGCAA GGTGAGGGTCACGAACACTT	59.31 59.89	50.0 55.0	144	(15)
β3-AR (Adrb3)	NM_013462.3	AGGCACAGGAATGCCACTCCAA GCTTAGCCACAACGAACACTCG	64.48 62.29	54.55 54.55	144	(30)
GAPDH	NM_001289726.1	AACACTGAGCATCTCCCTCA GTGGGTGCAGCGAACTTTAT	58.35 58.83	50.0 50.0	113	(31)

Tm: Melting Temperature; GC: guanine-cytosine content.

Relative gene expression

Figures 2, 3, and 4 show the relative gene expression of adrb3, Barr1, and Barr2 between study groups. The Adrb3 and Barr1 gene expressions did not significantly change between groups ($P > 0.05$) (Figures 2 and 3). However, the gene expression of β-Arrestin 2 was higher in HFD-OW group than in the control group ($P = 0.001$) and was lower in HFD-AT group when compared with OW-HFD group (Figure 4).

Discussion

The findings of this study include the following: First, HFD, which had a fattener effect, caused an increase in Barr2 gene expression. However, the HFD did not significantly change the expression of Barr1 and Adrb3
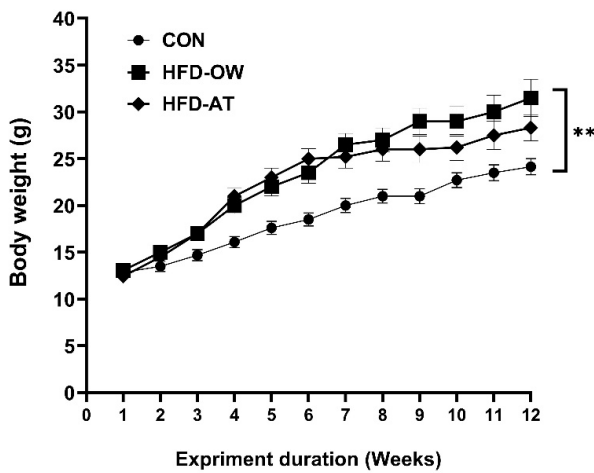
Figure 1. Bodyweight during the experiment period.
CON: Control (n=7); HFD-OW: High-fat diet-induced overweight; HFD-AT: a high-fat diet with aerobic training. Data are presented as mean±SEM. Data were analyzed by one-way ANOVA followed by Tukey's test. ** $P < 0.01$ compared to the control group.
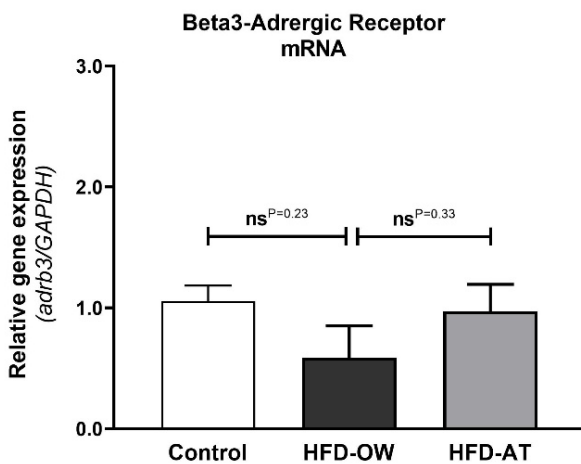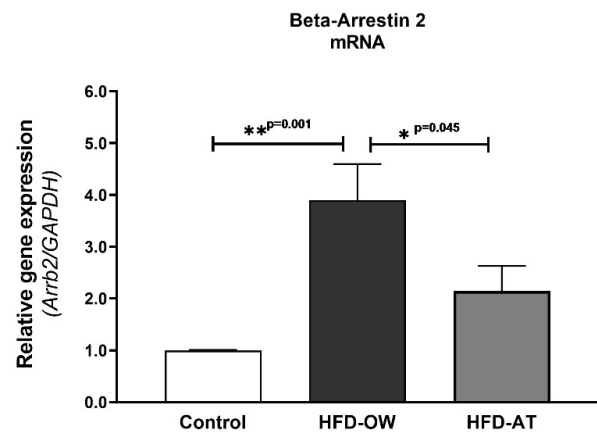
Figure 2. Relative gene expression of Beta3 adrenergic receptor (Adrb3) in the study groups.
HFD-OW: High-fat diet-induced overweight; HFD-AT: high-fat diet with aerobic training. Data are presented as mean±SEM. Data were analyzed by one-way ANOVA followed by Tukey's test.
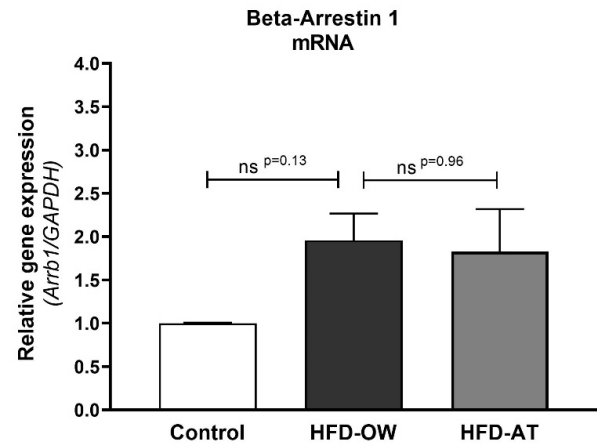
Figure 3. Relative gene expression of beta arrestin 1 (Arrb1) in the study groups.
HFD-OW: High-fat diet-induced overweight; HFD-AT: high-fat diet with aerobic training. Data are presented as mean±SEM. Data were analyzed by one-way ANOVA followed by Tukey's test.

Figure 4. Relative gene expression of beta arrestin 2 (Arrb2) in the study groups.
HFD-OW: High-fat diet-induced overweight; HFD-AT: high fat diet with aerobic training. Data are presented as mean±SEM. Data were analyzed by one-way ANOVA followed by Tukey's test.

genes. Second, the aerobic training significantly prevented the increase of Barr2 induced by the HFD. Nevertheless, the training did not significantly change the expression of Barr1 and Adrb3. Data showed that the post-experiment weight of the HFD-OW group was increased by 30% as compared to the control group. This suggested that the HFD had a fattener effect. Besides, they suggested that the HFD-OW group likely became obese/overweight. This suggestion was based on a review study reporting that the HFD-induced weight increase of 10% to 25% compared to the control group has been considered as moderate obesity (32). We cautiously hypothesized that the group was overweight. Further, the post-experiment weight of the HFD-AT group was 17% higher (but not significantly) than the control group. This might indirectly imply that the aerobic training prevented HFD-induced overweight. Notably, the Lee index was not used to estimate the degree of obesity in this study, because the index was examined

on rats and mostly applied to rats rather than mice (32,33). An important finding of this study was that the Barr2 gene expression was increased in HFD-OW group as compared to the control group. This was consistent with a previous study on humans showing that Barr2 gene expression was higher in subcutaneous WAT of the obese individuals as compared to age-matched non-obese ones (15). β-Arrs can suppress β-adrenergic signaling pathway by trafficking (internalization) mechanism (14). Therefore, these results raised the possibility that the β-adrenergic signaling may be impaired in adipose tissue as a result of HFD-induced overweight. However, in this study, we did not find a significant change in Barr1 expression in HFD-OW group as compared to the control group. This finding suggested that the transcription of the subtypes of Barr genes may be stimulated by different manners.

In contrast to the hypothesis, data did not show a significant reduction in Adrb3 gene expression in HFD-OW group compared to the control group. However, some previous studies have reported a downregulation in Adrb3 gene expression in WAT of obese rodents (8,9). This inconsistency might be due to two factors including the composition of HFD and the temperature of the environment in which mice were kept. In this study, HFD consisted of 45% fat, whereas, in the previous works, the amount of fat was 60%. In this study, mice were kept at 26°C, whereas, in previous studies, mice were kept at 22°C. Recent investigations have shown that the standard temperature (i.e., 22°C) can chronically induce light/cold stress in mice and may thus stimulate thermogenic mechanisms such as an expression of Adrb3 and uncoupling protein1 in WAT of mice (35,36). Overall, it was speculated that the HFD-induced overweight may impair the β3-adrenergic signaling by upregulating Barr2 gene expression. Sine β3-adrenergic signaling is essential for inducing catabolism (i.e., lipolysis and FFA oxidation) and thermogenesis (6,37), it was thought that the deficiency of β3-adrenergic signaling may be linked to dysregulation of fat oxidation and energy expenditure, probably developing obesity and its metabolic disorders.

Additionally, no significant change was observed in Adrb3 expression in HFD-AT group as compared to both HFD-OW and control groups. This finding was similar to that in a previous work by Rodrigues et al who reported that endurance training for eight weeks did not change in Adrb3 gene expression in WAT of mice (38). However, there are a few studies that reported an increase in Adrb3 expression following endurance training in WAT of rats that had a normal (21) and HFD (20). The reason(s) for the discrepancy is not clear. Nevertheless, this is perhaps related to rodent species. In the studies reporting the increase of Adrb3, subjects were rats, whereas, in the current study and the one conducted by Rodrigues et al, reporting no change in the expression of Adrb3, subjects were mice. Based on a few studies, Adrb3 expression may be differently affected in different rodent species. However, this needs further studies.

The main result of this study was that Barr2 gene expression was lower in HFD-AT group than in HFD-OW group. This result indicated that aerobic training could prevent the increase of Barr2 gene expression that was induced by HFD. Further, exercise was previously reported to acutely reduce the expression of Barr2 in WAT of mice (39). Considering that Barr2 can desensitize β-adrenergic signaling in adipocytes, this finding suggested that aerobic training may prevent the desensitization of β-adrenergic signaling by downregulating Barr2.

Conclusion
The gene expression of Adrb3 and Barr1 was not changed either by HFD or aerobic training in WAT of mice. However, the aerobic training could inhibit the upregulation of Barr2 gene expression that was induced by HFD. This indicated that aerobic training may prevent β-adrenergic-signaling deficiency by modifying the Barr2 expression. Further, it would be suggested that the partially preventive effect of aerobic training on the development of obesity and obesity-related diseases may be mediated by modulating the Barr2 expression.

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