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Original Article

Treadmill exercise increases cystathionine γ-lyase expression and decreases inflammation in skeletal muscles of high-fat diet-induced obese rats

Dongdong Wu[1], Zhen Lu[1], Mengling Li[1], Zhenxian Feng[2], Ailian Liu[2], Dongzhu Jin[1], Ailing Ji[1], Yanzhang Li[1]

[1]Henan University School of Medicine, Kaifeng 475004, Henan, China; [2]Henan University School of Physical Education, Kaifeng 475001, Henan, China

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Abstract: Obesity is a worldwide metabolic disorder that increases the risk of a number of human diseases. Physical exercise has many beneficial effects on obesity and related disorders, such as dyslipidemia and hypertension. The present study investigated the effects of treadmill exercise on the generation of endogenous hydrogen sulfide (H_2S) and the level of inflammation in skeletal muscles of high fat diet (HFD)-induced obese rats. Here we showed that the concentration of H_2S and the expression level of cystathionine γ-lyase (CSE) in skeletal muscles of HFD-fed rats were lower than those in skeletal muscles of low fat diet (LFD)-fed rats. However, treadmill running significantly increased the concentration of H_2S and the expression of CSE at both the mRNA and protein level in skeletal muscles of HFD-fed rats. Furthermore, the expression of interleukin-6 (IL-6) in the HFD group was higher than that in the LFD group; whereas, treadmill running reduced the IL-6 levels in skeletal muscles of HFD-fed rats. Moreover, no significant difference was observed in IL-15 expression between each group. In conclusion, treadmill exercise could enhance the concentration of H_2S by increasing CSE expression and decrease inflammation by reducing the level of IL-6 in skeletal muscles of HFD-fed rats.

Keywords: Treadmill running, hydrogen sulfide, cystathionine γ-lyase, inflammation, interleukin-6

Introduction

Obesity, which is an excessively high proportion of body fat, is a common metabolic disorder affecting over 500 million people worldwide [1-3]. Obesity is highly associated with the development of a number of human diseases, including cardiovascular disorders, type 2 diabetes, and non-alcoholic fatty liver disease [4]. Obesity is characterized by systemic, low-grade, chronic inflammation, which can be partly attributed to an increase in the proinflammatory systemic milieu imposed by proinflammatory cytokines [5, 6]. It has been demonstrated that proinflammatory adipocytokines, leptin, and C-reactive protein are elevated; however, adiponectin, the main insulin-sensitizing and anti-inflammatory compound, is lower in obesity [7]. A recent study showed that exercise training significantly improved muscle endurance and reduced markers of inflammation and oxidative stress in skeletal muscles of aged men [8]. Whether exercise training could ameliorate inflammation in high-fat diet (HFD)-induced obese rats needs further investigation.

Hydrogen sulfide (H_2S) is a colorless, toxic gas with a characteristic rotten-egg odor and is now widely considered to be an endogenous gaseous signaling molecule [9-11]. In mammals, endogenous H_2S is mainly generated from L-cysteine by the catalysis of two pyridoxal 5'-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), and by the combined action of cysteine aminotransferase and 3-mercaptopyruvate sulfotransferase (3-MST) [11-13]. Species specific differences have been observed with respect to the expression of CBS, CSE, and 3-MST in skeletal muscle [14]. A recent report suggested that CSE and CBS expression could be detected in human skeletal muscle, whereas there was no expression of these enzymes in mouse skeletal muscle [15]. Another study demonstrated that H_2S-generating enzymes (CSE, CBS, 3-MST) were present at detectable

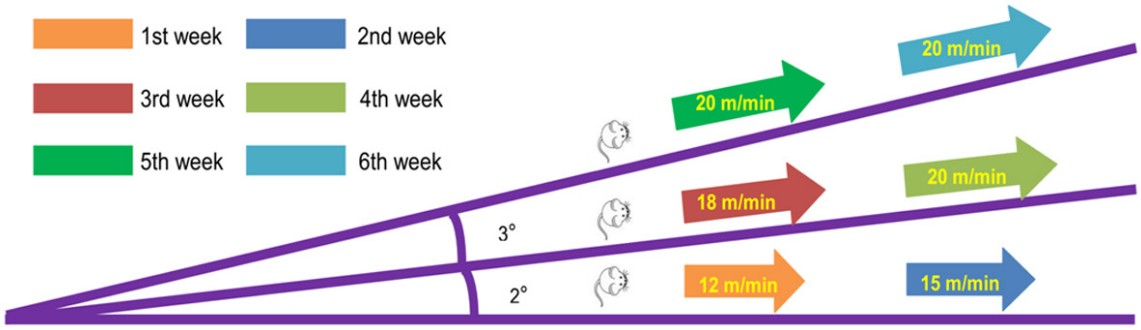

Figure 1. Proposed schematic diagram of weekly variation in treadmill speed and inclination of the exercise group.

levels in rat skeletal muscle [16]. However, whether exercise training could modulate the generation of endogenous H_2S in HFD-induced obese rats remains to be fully elucidated.

In the present study, the effects of treadmill running on the levels of endogenous H_2S and inflammation in skeletal muscles of HFD-induced obese rats were investigated. Our results showed that treadmill running increased the expression of CSE and decreased the level of IL-6 in skeletal muscles of HFD-induced obese rats.

Materials and methods

Animals

All procedures for animal experiments were approved by the Committee of Medical Ethics and Welfare for Experimental Animals of Henan University School of Medicine. Five-week-old male SD rats were purchased from The Experimental Animal Center of Zhengzhou University (Henan, China). Rats were maintained in a controlled environment (temperature 22 ± 3°C and humidity 55 ± 5%) on a 12-hour light/dark cycle with food and water *ad libitum*. The rats were fed either a low fat diet (LFD, 10% kcal as fat, Medicience Ltd., Jiangsu, China) or high fat diet (HFD, 45% kcal as fat, Medicience Ltd., Jiangsu, China) for a total of 14 weeks. After 8 weeks of feeding, the HFD-fed mice were randomly assigned to the HFD group (n = 10) or the HFD with exercise training (EX) group (HFD + EX, n = 10). These mice were treated for an additional 6 weeks. EX consisted of running for 40 minutes, 5 days per week, as described in **Figure 1**. The LFD group (n = 10) continued with the LFD for the entire 6-week duration. The body weight gain, food intake, and calorie consumption in rats were measured. At the end of the experiment, the rats were killed and the gastrocnemius muscle was stored at -80°C for further study.

RNA extraction and RT-PCR

Total RNA was isolated from skeletal muscle using TRIzol reagent (Life Technologies, Rockville, MD, USA), treated with DNase I (Roche Applied Science, Mannheim, Germany), and purified using an RNA clean up kit (Cwbiotech, Beijing, China). One microgram of total RNA was applied for cDNA synthesis using a cDNA reverse transcription kit (Cwbiotech, Beijing, China). Primers were designed with Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA): CBS, forward 5'-CTCC-GGGAGAAGGGTT-TTGA-3' and reverse 5'-CATGTTCCCGAGAG-TC-ACCAT-3'; CSE, forward 5'-GCTGAGAGCCTGG-GAGGATA-3' and reverse 5'-TCACTGATCCCGA-GGGTAGCT-3'; 3-MST, forward 5'-CG-GCGCT-TCCAGGTAGTG-3' and reverse 5'-CTGGTCAG-GAATTC-AGTGAATGG-3'; IL-6, forward 5'-TAT-GAACAGCGATGATGCAC-TG-3' and reverse 5'-TTGCTCTGAATGACTCTGGCTT-3'; IL-15, forward 5'-GACAGTGACTTTCATCCCAGTT-3' and reverse 5'-CATT-CCTTGCAGCCAGAC-3'; and β-actin, forward 5'-ACCCGCGAGT-ACAACCTTCTT-3' and reverse 5'-TATCGTCATCCATGGCGAACT-3'. The PCR reactions were performed in a total volume of 25 μL using the following thermal cycling parameters: 95°C for 5 min, 35 cycles of 93°C for 30 s, 60°C for 45 s, and 72°C for 5 min. The mRNA expression levels of the test genes were normalized to β-actin levels.

Western blot analysis

For western blot analysis, frozen muscle samples (50 mg) were powdered under liquid N_2 and homogenized in 200 μL of ice-cold RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). Skeletal muscle protein concentrations were measured with a BCA pro-

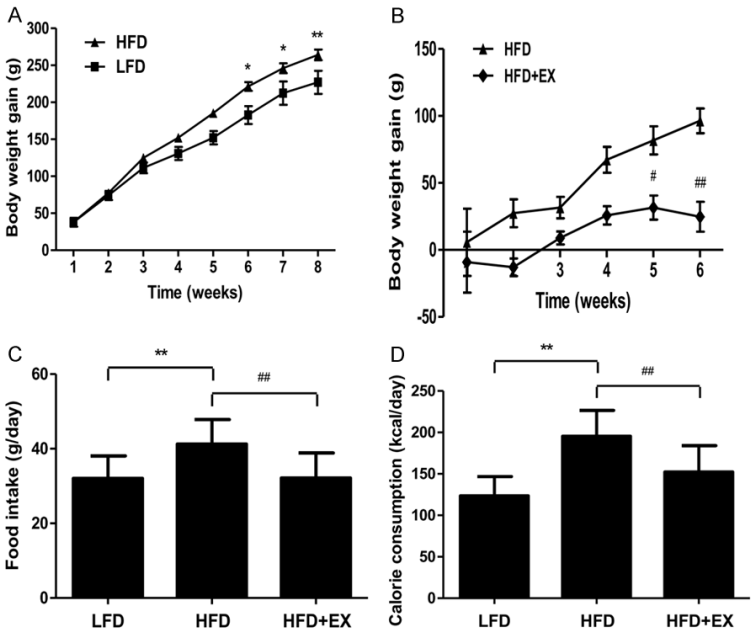

Figure 2. Effect of treadmill running on the body weight gain, food intake, and calorie consumption in rats. A. The body weight gain of rats in the LFD and HFD groups. B. The body weight gain of rats in the HFD and HFD + EX groups. C. Food intake. D. Calorie consumption. Values are presented as the mean ± SD (n = 10); $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LFD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the HFD group.

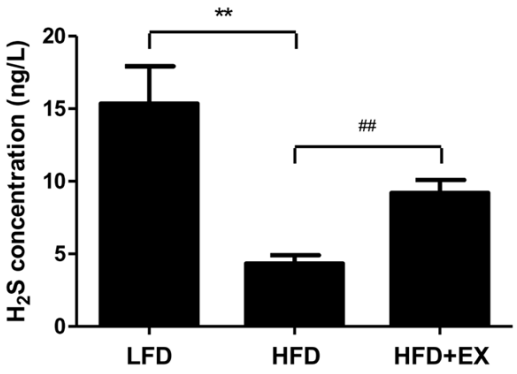

Figure 3. Effect of treadmill running on H_2S concentration in rat skeletal muscles. Values are presented as the mean ± SD (n = 3); $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

tein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The extracted proteins (40 μg) were separated on an SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with an anti-CBS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-3-MST antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CSE antibody (Protein Tech Group, Chicago, IL, USA), and anti-β-actin (Protein Tech Group, Chicago, IL, USA). The membranes were thoroughly rinsed and then incubated with a horseradish peroxidase-conjugated secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China). The reaction was visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific, Rockford, IL, USA). Immunoblots were quantified by densitometry using Quantity One software (Bio-Rad, CA, USA).

Measurement of H_2S concentration

To investigate the effect of treadmill running on the production of H_2S and release in skeletal muscles (50 mg), we performed enzyme-linked immunosorbent assay (ELISA) by using a commercial ELISA kit (Antibodies-online Inc., Atlanta, GA, USA) according to the manufacturer's instructions.

Statistical analysis

All data were presented as the mean ± standard deviation (SD). The expression levels of mRNA and protein were normalized to a calibrator which was considered as the basal sample. Significant differences between groups were analyzed by two way analysis of variance using SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA), followed by Tukey's test. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Treadmill running reduces the body weight gain, food intake and calorie consumption in HFD-fed rats

As shown in **Figure 2A**, in comparison to rats fed with a LFD for 8 weeks, HFD-fed rats exhibited increased body weight gain, which indicated that an alimentary obesity model had been successfully established. It has been widely

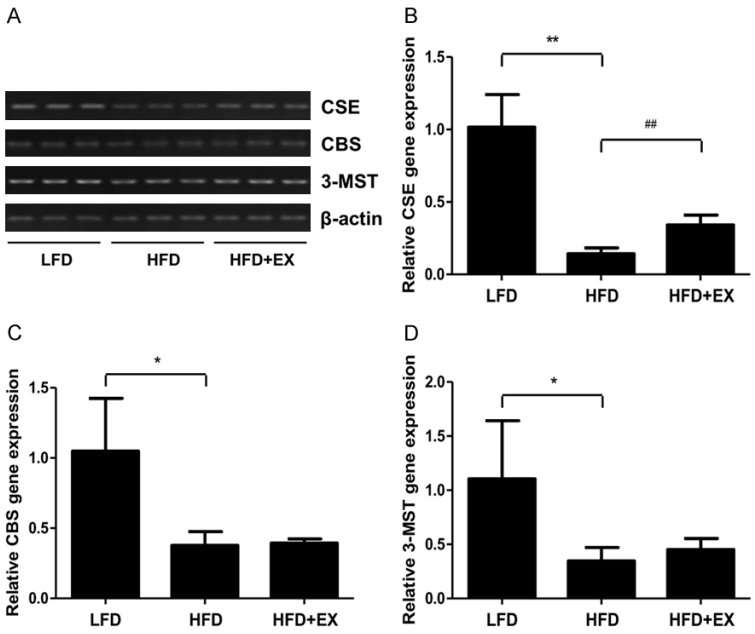

Figure 4. Effect of treadmill running on the mRNA levels of CSE, CBS, and 3-MST in rat skeletal muscles. (A) The expression levels of CSE, CBS, and 3-MST were measured by RT-PCR. β-actin was used as an internal control. Bar graphs showed the quantification of CSE (B), CBS (C), and 3-MST (D). Values are presented as the mean ± SD (n = 3); $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

Treadmill running increases CSE gene expression in skeletal muscles of HFD-fed rats

Considering that CSE, CBS, and 3-MST are the main H_2S-generating enzymes in mammals, we investigated the genetic expression of H_2S-generating enzymes in rat skeletal muscles. The results suggested that the genetic expression of CSE, CBS, and 3-MST in skeletal muscles of HFD-fed rats were lower than those in skeletal muscles of LFD-fed rats (**Figure 4A-D**), while treadmill running observably increased CSE gene expression in skeletal muscles of HFD-fed rats (**Figure 4A** and **4B**).

Treadmill running increases CSE protein expression in skeletal muscles of HFD-fed rats

We further measured the protein expression of CSE, CBS, and 3-MST in rat skeletal muscles. The results showed that the protein levels of CSE in the skeletal muscles of HFD-fed rats were lower than those in skeletal muscles of LFD-fed rats (**Figure 5A** and **5B**). After 6 weeks of treadmill running, the protein expression of CSE in skeletal muscles of HFD-fed rats was significantly increased (**Figure 5A** and **5B**). Furthermore, there were no significant differences in the protein levels of CBS and 3-MST between each group (**Figure 5A**, **5C** and **5D**).

accepted that exercise training has beneficial health effects on the prevention of obesity and related disorders, such as dyslipidemia and hypertension [17-19]. The results showed that treadmill running for 6 weeks significantly reduced the body weight gain in rats fed with HFD (**Figure 2B**). Compared to the LFD-fed rats, HFD-fed rats showed increased food intake and calorie consumption, which were all reversed by tread mill running (**Figure 2C** and **2D**).

Treadmill running increases the H_2S concentration in skeletal muscles of HFD-fed rats

A recent study found that obesity in mice reduced the bioavailability of H_2S and the intracellular concentration of H_2S was lower in adipose tissue macrophages (ATMs) isolated from obese mice compared to ATMs from lean mice [20]. Our results indicated that the concentration of H_2S in skeletal muscle of HFD-fed rats was lower than that in skeletal muscles of LFD-fed rats. However, after 6 weeks of treadmill running, the H_2S concentration in skeletal muscles of HFD-fed rats was markedly increased (**Figure 3**).

Treadmill running reduces IL-6 gene expression in skeletal muscles of HFD-fed rats

IL-6 plays an important role in the regulation of appetite, body composition, and energy expenditure [21, 22]. As shown in **Figure 6A** and **6C**, the genetic expression of IL-6 in skeletal muscles of HFD-fed rats was higher than that in skeletal muscles of LFD-fed rats. Treadmill running significantly reduced IL-6 gene expression in skeletal muscles of HFD-fed rats. IL-15 is an anabolic factor that is highly expressed in skeletal muscles. Recent studies have shown

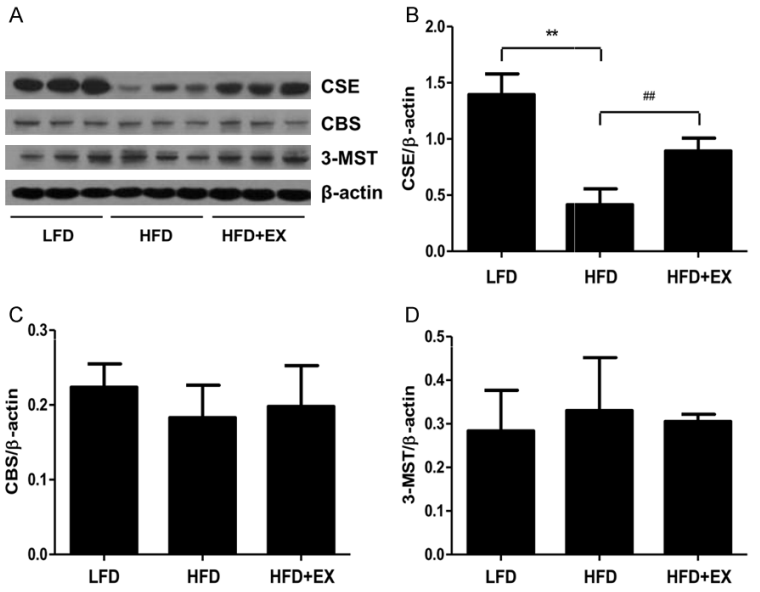

Figure 5. Effect of treadmill running on the protein levels of CSE, CBS, and 3-MST in rat skeletal muscles. (A) The expression levels of CSE, CBS, and 3-MST were measured by western blot. β-actin was used as an internal control. Bar graphs show the quantification of CSE (B), CBS (C), and 3-MST (D). Values are presented as the mean ± SD (n = 3); $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

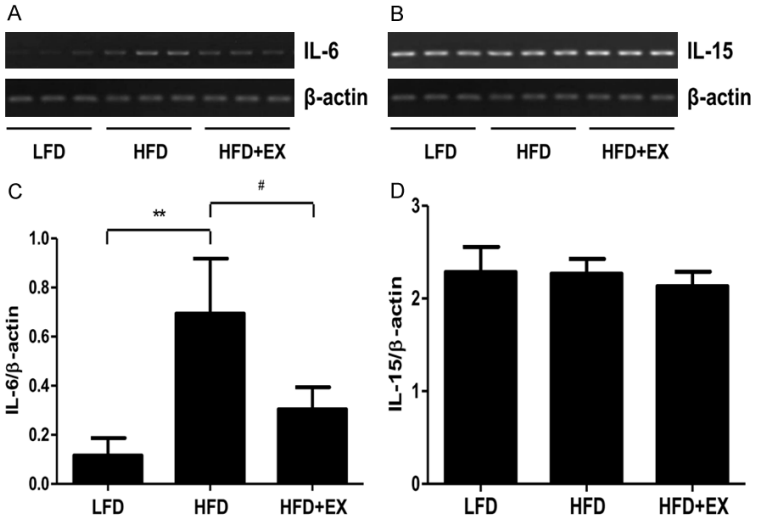

Figure 6. Effect of treadmill running on the mRNA levels of IL-6 and IL-15 in rat skeletal muscles. (A and B) The expression levels of IL-6 and IL-15 were measured by RT-PCR. β-actin was used as an internal control. Bar graphs show the quantification of IL-6 (C) and IL-15 (D). Values are presented as the mean ± SD (n = 3); $^{**}P < 0.01$ compared with the LFD group; $^{\#}P < 0.05$ compared with the HFD group.

that IL-15 is involved in skeletal muscle fiber growth, glucose uptake, and hypertrophy [23-25]. Our results showed that there was no significant difference in IL-15 gene expression between each group (**Figure 6B** and **6D**).

Discussion

It is widely accepted that CSE, CBS, and 3-MST are the three main H_2S-generating enzymes in mammals. These enzymes could be detected in different organs and tissues, and their expression levels are significantly different [26, 27]. CSE is mainly expressed in the cardiovascular system, while CBS is abundant in the central nervous system. Furthermore, 3-MST and CAT are apparently important for H_2S production in the vasculature and brain [9, 12, 28]. To explore the effect of treadmill running on the existence of the skeletal muscle-derived endogenous H_2S pathway in HFD-fed rats, we detected the H_2S content and the expression of H_2S-generating enzymes in rat skeletal muscles. Our results showed that CSE, CBS, and 3-MST could be detected at the mRNA and protein levels in skeletal muscles of both LFD- and HFD-fed rats, which is consistent with the findings of a recent study [16]. Furthermore, the H_2S concentration and the mRNA and protein levels of CSE in skeletal muscles of HFD-fed rats were lower than those in skeletal muscles of LFD-fed rats, while treadmill running increased the concentration of H_2S and mRNA and protein levels of CSE in skeletal muscles of HFD-fed rats. These results indicate that CSE is the major H_2S-generating enzyme in rat skeletal muscles and treadmill running could enhance the concentration of H_2S by increasing CSE expression in skeletal muscles of HFD-fed rats.

Obesity is coupled to a low-grade, chronic inflammatory state characterized by activation in

adipose tissue, immune cell infiltration, and systemic elevation of pro-inflammatory cytokines [5, 29, 30]. IL-6 is a novel adipokine that is expressed by the adipocytes and stromovascular matrix of visceral white adipose tissues involved in the development of obesity [18]. Recent studies have shown that the IL-6 concentration in plasma is increased with weight gain and decreased upon weight loss [31, 32]. We observed that the IL-6 level in the gastrocnemius muscle of HFD-fed rats was higher compared to the LFD group, suggesting that IL-6 plays an important role in obesity-associated inflammation. Recent findings indicate that exercise training-induced adaptations in adipose tissue metabolism are mediated by IL-6, which is secreted from skeletal muscles during exercise [33-35]. In this study, we found that treadmill running significantly reduced the IL-6 level in skeletal muscles of HFD-fed rats. These data demonstrate that the anti-inflammatory effect of treadmill running could be achieved, at least in part, by reducing the expression of IL-6 in the gastrocnemius muscle of obese individuals.

IL-15 is a recently discovered pleiotropic cytokine that belongs to the four α-helix bundle family of cytokines that are known to enhance natural killer cell activity and stimulate T cell proliferation [25, 36]. A recent study showed that the expression level of IL-15 was lower in the gastrocnemius muscle of HFD-fed rats than in controls [25]. In contrast, another study indicated that there was no significant difference in the level of IL-15 protein in the gastrocnemius muscle between Zucker diabetic fatty (ZDF) and ZDF lean control rats [23]. In our study, no remarkable difference was observed in the gene expression of IL-15 in the gastrocnemius muscle between the LFD and HFD groups. Recently, there has been controversy regarding the effects of exercise training on muscle IL-15 protein levels and mRNA expression [37]. For instance, the mRNA expression of IL-15 in skeletal muscle has been shown to increase in response to acute resistance exercise in fast skeletal muscles [38]. However, another study suggested that chronic low frequency/low volume resistance training did not change the IL-15 mRNA level in plantaris muscles, despite the induction of hypertrophy [39]. Similarly, we found that treadmill running had no effect on the IL-15 mRNA level in the gastrocnemius muscle of HFD-fed rats. Taken together, there was no significant difference in the expression of IL-15 in the gastrocnemius muscle between LFD and HFD-fed rats, and treadmill running did not affect the expression level of IL-15 in the gastrocnemius muscle of HFD-fed rats.

In conclusion, treadmill exercise could enhance the concentration of H_2S by increasing the expression level of CSE and could decrease inflammation by reducing the expression of IL-6 in skeletal muscles of HFD-fed rats. Therefore, treadmill exercise may possess therapeutic potential in treating obesity-induced inflammation in obese individuals.

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Disclosure of conflict of interest

None.

Address correspondence to: Yanzhang Li and Ailing Ji, Henan University School of Medicine, Kaifeng 475004, Henan, China. Tel: +86 371 23880585; Fax: +86 371 23880585; E-mail: yanzhang206@163.com (YZL); ailingji@163.com (ALJ)

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