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ORIGINAL PAPER

The Effects of Resveratrol, Metformin, Cold and Strength Training on the Level of Perilipin 5 in the Heart, Skeletal Muscle and Brown Adipose Tissues in Mouse

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Abstract
The high accumulation of lipid droplets in the cell is related to metabolic disorders, such as obesity. Perilipin 5 (Plin5), plays an important role in triglyceride hydrolysis in the lipid droplets. In this study, this protein has been evaluated in different tissues and conditions in mice. Fifty male mice were divided into 5 groups and treated for 45 days with Resveratrol, Metformin, strength training, and 4 °C cold. Brown adipose tissue (BAT), gastrocnemius skeletal muscle and heart were isolated for RNA extraction. The Plin5 gene expression was evaluated, using Real-Time PCR, and the plin5 was analyzed at the protein level, using western blot. In BAT, Resveratrol significantly reduced the plin5 protein level and gene expression ($p < 0.05$). In heart tissue, Resveratrol and strength training, decreased ($p < 0.05$) the plin5 expression, but Metformin increased the gene expression ($p < 0.05$). In skeletal muscle, resveratrol, strength training, cold and Metformin significantly increased the plin5 expression at the gene and protein level ($p < 0.05$). In BAT, Resveratrol has a greater effect in decreasing lipid deposits, compared with the strength training and cold; thus, it can play a better role in preventing lipid accumulation. In heart tissue, Resveratrol probably decreases insulin resistance, due to the increased expression of plin5 in skeletal muscle.

Keywords Resveratrol · Metformin · Strength Training · Cold · Perilipin-5

Introduction

Obesity is identified as the greatest health problem worldwide, with an increase in lipid accumulation in body tissues, and is characterized by a BMI > 30 [1, 2]. Lipid droplets (LD) play an important role in the metabolism and energy balance in mammalian cells. There are more than two hundred types of proteins in LD, most of which are perilipins (plins), playing an important role in stabilizing, degenerating and transferring of LD. The high accumulation of LD in the cell is related to metabolic disorders, such as obesity, diabetes, and atherosclerosis [3, 4].

Plin5 or OX/PAT is more involved in tissues, such as the heart, skeletal muscle, and brown adipose tissue (BAT), taking part in the oxidation of fatty acid. This protein acts around LD as scaffolds, and plays an important role in lipolysis. Plin5 serves as a barrier by decreasing lipolysis via PPARα, especially in the heart tissue, [5] and plays a unique role in utilizing fatty acids' oxidation, via PGC1α [6, 7]. In addition, it plays a significant role in skeletal muscle in lipolysis of fat droplets, and any impairment in its function results in insulin resistance in skeletal muscle, leading to ceramide accumulation [8].

Resveratrol is a polyphenol composition that exists in red grape, peanut, berry, and other herbal compounds, having anti-inflammatory and anti-oxidant effects and improving glucose tolerance [9, 10]. Resveratrol also increases lipolysis, decreases the synthesis of fatty acids and increases fatty acid esterification in mitochondria [11]. Furthermore,

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Resveratrol prevents the differentiation of progenitor lipid cells (3T3-L1), into adult fat cells by reducing PPA R-γ [12].

Cold causes an increase in PGC1α that is regulated and controlled by PPARɣ and *plin5 [*13]. Metformin reduces the activity of acetyl-coA carboxylase and increases oxidation via AMPK in the liver cells [14].

Increasing the size and number of LD causes' obesity, increases insulin resistance and cardiovascular problems; plins family, especially plin5, plays an important role in controlling and regulating hydrolysis of LD, especially in the cardiac tissue and oxidative tissues, such as BAT and skeletal muscle. Therefore, this study aimed to examine the effects of Resveratrol, Metformin, and cold and strength training on the *plin5* (mouse) expression in the indicated tissues, in order to pave the path for controlling obesity and its side effects.

Materials and Methods

Animals: In this experimental study, fifty C57BL/6 J male mice, 6–8 weeks-old, weighing 20–25 grams, were randomly divided into five groups. The study was performed, in accordance with the standard animal study guideline, and all experimental protocols were approved by the Shahre-Kord University of Medical Sciences Ethics Committee (IR.SKUMS.REC.1395.195). For 45 days, the first group received DMSO, the second group 400 mg/kg of Resveratrol dissolved in DMSO, and the third group received Metformin 250 mg/kg, via gavage. The fourth group performed high-intensity strength training, using a treadmill [15]. The fifth group was initially kept at 18 °C for a week, and then kept at 4 °C, until the end of the experiment (45 days). After 45 days the mice were anesthetized with chloroform and then the subclavicular BAT, gastrocnemius muscle, and heart were isolated from the mice. RNA extraction was performed, using Trizol (Ambion TRIZOL reagent) and the quality and purity were investigated with a Nanodrop (Thermo scientific 2000 spectrophotometer, USA). DNase was used to remove potential DNA in the reaction and then cDNA were synthesized, using the commercial kit. RNA extraction and cDNA synthesis were performed on ice bath. The *plin5* gene expression was measured with the Rotor-Gene Q 3000 (Australia), against 18 s RNA gene, using commercial kits, containing SYBR green (Fermentas Thermo Scientific, Canada). The primer sequences used in this study are presented in Table 1.

The amplification was carried out, using the following conditions: initial enzyme activation at 94 °C for 5 min, then 40 cycles of 95 °C for 15 s, 61.5 °C for 20 s, and 72 °C for 30 s. The quantitation of the data was performed, using the comparative CT ($2^{-\Delta\Delta CT}$) method.

Table 1 Primer sequences and product lengths

Genes	Primer Sequences (5′ → 3′)	Amplicon Length (bp)
Plin5	F: GGTGACATCAGCCAAGGATACAG R: TCCACCAGCTTCTCCGACTT	147
18s RNA	F: CGCAAATTACCCACTCCCGAC R: GTAACCTCCCGTTCAGACCAC	133

For western blots, first BAT, gastrocnemius muscle and heart were homogenized in RIPA Lysis buffer, containing 50 mM Tris Hcl, 150 Mm NaCl, 1%TritonX-100, 1Mm EDTA, and 0.1% SDS 1 mM and then protein concentrations were determined by the Bradford method [16]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference protein.

Proteins were separated on 12% SDS PAGE, transferred to a PVDF membrane, incubated with blocking buffer, containing 5% Bovine Serum Albumin (BSA), and then washed and incubated with primary antibody (anti-Perilipin 5 (C-terminus) guinea pig polyclonal antibody), (GP31, Progen biotechnik, Heidelberg, Germany), as indicated overnight at 4 °C. Subsequently, the membrane was washed and incubated with rabbit anti-guinea pig polyclonal secondary antibody HRP (IgG H&L) (ab6771, Abcam, Cambridge, UK) at room temperature for 2 h, and then the protein band was visualized, using the ECL kit, and scanned on a LICOR Biotechnology system.

Statistical analysis

Statistical analyses were performed, using non-parametric Kruskal–Wallis test, and the pair comparisons between the groups were performed by Mann–Whitney test. All statistical analyses were performed, using Graph Pad Prism software (v 6.01), and data were presented as mean ± SD. All experiments were performed in triplicate and $p < 0.05$ was considered significant.

Results

At the end of the study, the weight gain of the Metformin, strength training, and cold groups was lower than the control group ($p = 0.01$, 0.03, 0.03, respectively), but no significant change has been found in the Resveratrol receiving group, compared to the control group ($p = 0.08$).

In BAT, the *plin5* expression was significantly decreased by 3.2 fold, compared to the control group, using Resveratrol ($p = 0.02$). The *plin5* expression in the Metformin and strength training groups increased by 2.3 and 1.8 fold, respectively, compared to the control (Fig. 1a). The plin5 protein level also significantly decreased by 2.43 fold, in

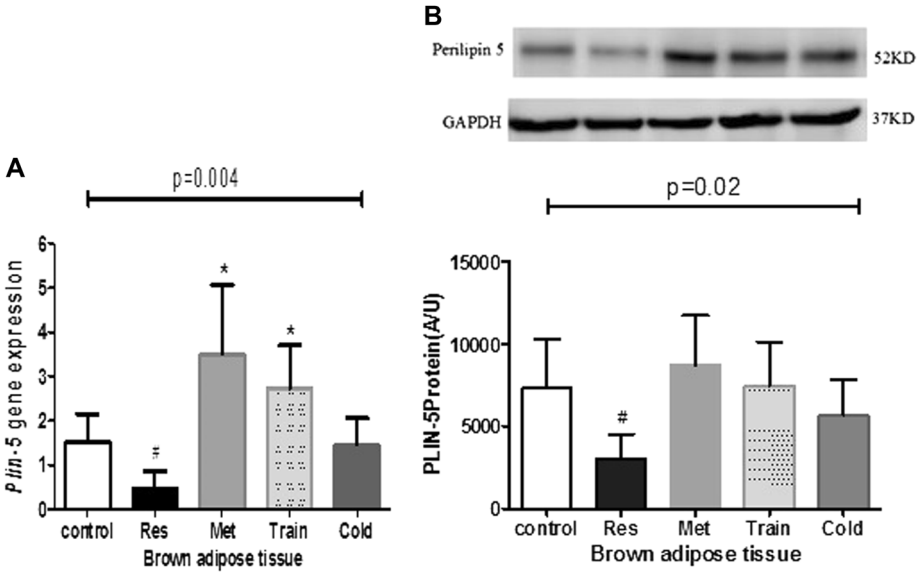

Fig. 1 The effects of Res (Resveratrol) 400 mg/kg/day, Met (Metformin)/250 mg/kg, Cold and Train (Strength training) on the *plin5* expression and Perilipin 5 protein content in BAT. **a** Real-time PCR **b** western blot analysis. Asterisk, Significant increase, compared to the control group, Hash, Significant decrease, compared to the control group

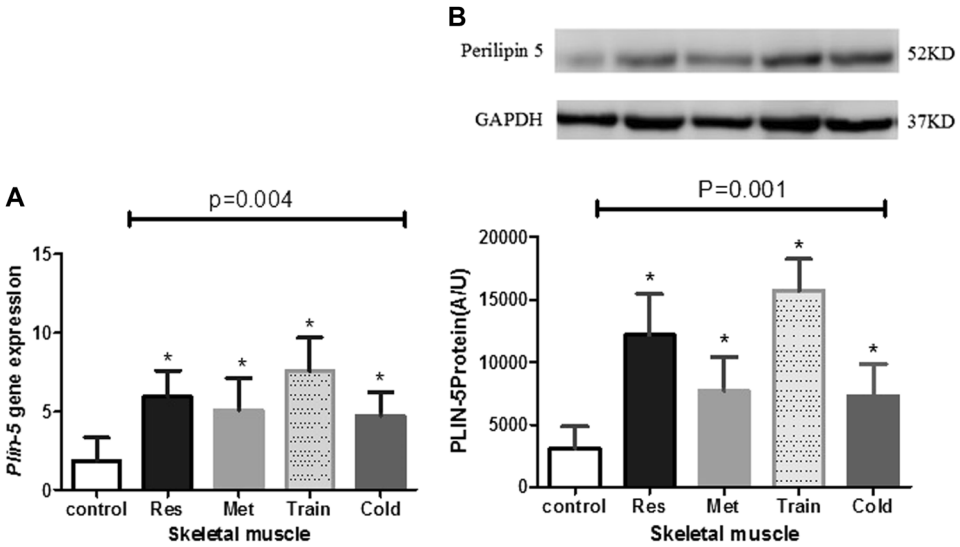

Fig. 2 The effects of Res (Resveratroll) 400 mg/kg/day, Met (Metformin)/250 mg/kg, Cold and Train (Strength training) on the *plin5* expression and perilipin 5 protein content in skeletal muscle. **a** Real-time PCR **b** western blot analysis. Asterisk, Significant increase, compared to the control group

Resveratrol receiving group ($p = 0.03$), but no significant change has been found in Metformin, strength training, and cold groups, compared to the control group ($p = 0.19$, 0.3, 0.2, respectively) (Fig. 1b).

In skeletal muscle, the *plin5* expression increased by 3.21 ($p = 0.005$), 2.69 ($p = 0.03$), 4.08 ($p = 0.002$), and 2.5 fold ($p = 0.05$), using Resveratrol, Metformin, strength training, and cold, respectively, compared to the control group (Fig. 2a). The plin5 protein level, (showed in Fig. 2b) increased in four treatment groups versus the control group (Resveratrol, 3.94 fold ($p = 0.0018$), Met (metformin), 2.45 fold ($p = 0.02$), train (Strength training), 5.09 fold ($p = 0.002$), and cold, 2.35 fold ($p = 0.046$).

In heart tissue, the *plin5* expression was significantly decreased, by 1.89 fold ($p = 0.046$), and 5.3 fold ($p = 0.005$) by Resveratrol and strength training, respectively, but Metformin slightly increased the gene expression level by 1.84 fold, compared to the control group ($p = 0.048$). The *plin5* expression did not significantly change, in cold-treated group (1.46 fold, $p = 0.3$) (Fig. 3a).

In heart tissue, the plin5 protein level (Fig. 3b) was decreased by 1.71 and 2.9 fold ($p = 0.02$, $p = 0.01$), in Resveratrol and strength training groups, respectively, compared to the control. No significant change has been found in protein content in heart tissue, after treatment with Metformin and cold, compared to the control group ($p = 0.08$ and $p = 0.3$, respectively).

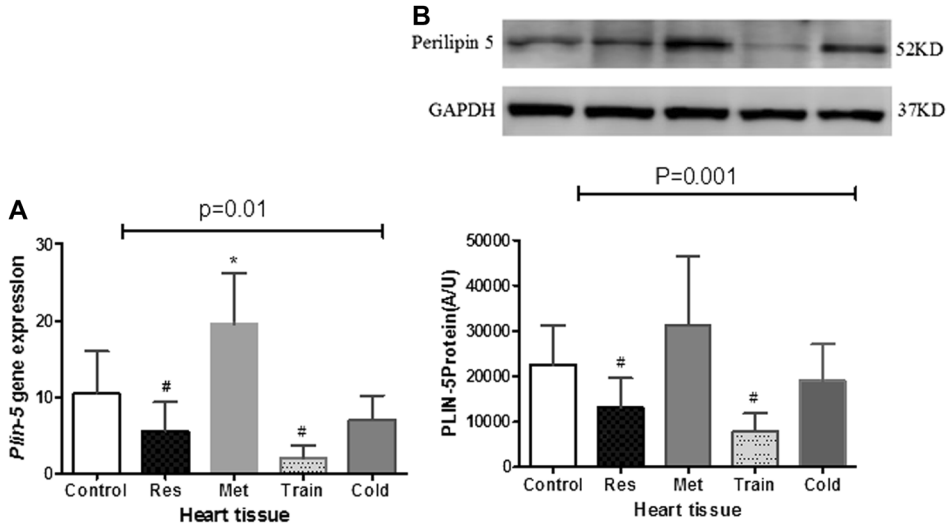

Fig. 3 The effects of Res (Resveratroll) 400 mg/kg/day, Met (Metformin)/250 mg/kg, Cold and Train (Strength training) on the *plin5* expression and perilipin 5 protein content in heart muscle. **a** Real-time PCR **b** western blot analysis. Asterisk, Significant increase, compared to the control group, Hash, Significant decrease, compared to the control group

Discussion

LD play a major role in the metabolism of tissue lipids, and plin5 plays an important role in lipolysis of LD, leading to changes in lipid flux. BAT can interfere with obesity, due to its important role in thermogenesis. Resveratrol affects the morphology of LD in rat liver cells, and reduces the expression of plins family, such as plin2 and TIP47; thus reduces the triglyceride content of LD and prevents the lipid accumulation in the liver [17]. The reduction of plin5, reported in this study, is consistent with the significant reduction of plin5 in BAT in our study. Therefore, Resveratrol by reducing plin5 in BAT, induce lipolysis in LD.

Cold is an inducing factor in the browning of adipose tissue. Cold affects BAT, through the sympathetic nervous system and beta-3 adrenergic receptor, by activating protein kinase A, leading to an increase in the factors involved in the thermogenesis, including UCP-1, PPARβ1, PGC1α, and PPARα [18]. Jin et al. (2015) showed that cold increases plin2, after 72 h in BAT, and slightly increases the OXPAT (PLIN5) after 24 h, and then a slight decrease after 72 h was reported, while exposing with cold (4 °C) in rats [19]. In the present study, long-term cold did not alter plin5 in BAT, which is probably due to the adaptation of rats to cold temperature.

Strength trainings increase perilipin 5 in BAT, probably due to the important role that have in the synthesis and function of mitochondria, as well as lipolysis and lipogenesis of LD. In 2016, Ramos et al. showed that strength trainings (using a treadmill), increased the level of plin5 proteins, in the subclavicular BAT of Sprague rats, relative to untrained rats, thereby decreasing the amount of acetyl-coA carboxylase protein in BAT [20]. In the present study, strength training in BAT only increased the expression of the *plin*, but no significant change has been found in the level of plin5 proteins, and this finding is in contrast with the study of Ramos et al. This inconsistency is probably due to the type of animal, used in the study or a consequence of the design of studies.

Metformin in rats with high-cholesterol diet increases AMPK by phosphorylation of plin, leading to lipolysis [21]. In our study, Metformin did not produce a significant change in the plin5 protein level in BAT, so it may have led to lipolysis, by altering the level of plin5 phosphorylation.

The heart is surrounded by lipids from plins family, especially plin5, acting as a barrier to LD against ATGL and lipases. The *plin5* acts via a PPARα transcription factor, causing a negative relationship between the levels of intra myocardial TAG and the cardiac function. Chronic lipid accumulation also negatively affects the heart function [22]. Myocardial steatosis is associated with coronary heart diseases, so there is an association between increased LD and heart diseases. Plin5 is an important marker for controlling lipolysis in the heart, its excessive increase, causes cardiac steatosis and cardiac hypertrophy [23, 24].

In our study, Resveratrol and strength training have reduced the *plin5* expression, as well as decreased the level of plin5 protein. Therefore, it is expected to act as a protective factor, against increased lipids of the heart, by modulating and increasing the lipolysis, leading to a decrease in heart triglyceride content.

Plin5 reduces myocardial ischemia by reducing oxidative stress, and prevents lipolysis of LD. An increase in lipolysis in rats, lacking *plin5*, followed by an increase in ROS (Reactive Oxygen Species), leads to heart malfunction. In fact, the relative expression of *plin5* in the heart is required for energy storage [25]. According to this study, Metformin can partly prevent the excessive reduction of plin5. This function of Metformin is probably due to the sugar burning

rather than lipid, which is associated with less oxidative stress.

Strength training increases plin2 and plin5, increasing intramyocellular triacylglycerol (IMTG), insulin sensitivity, activity of protein kinase A (PKA), and increasing the expression of hormone-sensitive lipase. In fat rats with high-fat diets, increasing intramyocellular lipid (IMCL) also increases insulin resistance [26].

Plin5 is an important protein in the LD, helping in turnover of lipids [27]. Cold with increasing PGC1α in skeletal muscle plays an important role in the synthesis of mitochondria; plin5 also plays an important role in regulating PGC1α, therefore, the cold probably affects mitochondria by changing the plin5 and PGC1α. Plin5 promotes the transfer of LD content to mitochondria and optimizes the oxidation of LD. In addition, plin5 regulates the hydrolysis of LD, in order to protect the mitochondria against sudden changes in the density of free fatty acids [28]. The association between IMCL and sensitivity to insulin is complex. IMCL increases insulin sensitivity; in obese people, with increasing IMCL, insulin resistance also increases; this process has been observed as a paradox in athletes. Training is associated with an increase in IMCL, but unlike obese people, increased insulin sensitivity has also seen in athletes. Increased sensitivity to insulin depends on the IMCL content [26].

In our study, Resveratrol, Metformin, strength training, and cold increased the *plin5* at the gene and protein levels, in skeletal muscle. However, the effect of Resveratrol, especially high-intensity strength training, is more prominent.

Resveratrol increases SIRT1 protein levels in skeletal muscle cells [29]. SIRT1 as a deacetylase controls PGC-1α gene expression [30] and SIRT1/ PGC-1α pathway can directly regulate the biophysiological functions of skeletal muscle. On the other hand, during stimulation of cells or tissues by exercise and cold exposure, plin5 is phosphorylated by protein kinase A and activates SIRT1/ PGC-1α pathway [31]

We anticipate that the observed changes in this study in all four groups' lead to higher IMTG production and more lipid accumulation in the skeletal muscle, followed by increased insulin sensitivity.

Conclusion

Plin5 is important because of the unique properties and different roles that it plays in BAT, skeletal, and cardiac muscle. Resveratrol reduces plin5 in BAT, accelerating the lipid hydrolysis in BAT. Therefore, it likely leads to a weight loss. In heart, Resveratrol and strength training reduce plin5 and can therefore lead to a decrease in myocardial steatosis. In skeletal muscle, Resveratrol, strength training, cold, and Metformin have been shown to increase plin5. An increase in perilipin-5 in the skeletal muscle may reduce insulin resistance.

The findings of this study help to understand the features and implications of plin5 in various tissues and the important role of this protein in obesity-related diseases.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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