Correlations between Plasma Sphingosine-1-phosphate (S1P) and Gene Expression of S1P Receptors with Mogenic Regulatory Factors Following Resistance Training

Ebrahim Banitalebi 1, Reza Gharakhanlou 2

1 Assistant professor in exercise physiology, University of Shahrekord. Shahrekord, Iran.
2 Associate professor in Exercise Physiology, Department of Sport Sciences, Tarbiat Modares University, Tehran, Iran.

Abstract
Background: The purpose of present study was to investigate whether Sphingosine 1-phosphate (S1P) levels and its receptors gene expressions are correlated with MyoD and myogenin following resistance training.

Materials and Methods: 24 eight-week-old male Wistar rats (190-250 gr) were randomly assigned into a control (n=12) or training (n=2) group. The rats climbed a resistance training ladder with weights attached to their tails. The content of plasma S1P and relative mRNA expression was determined by high performance liquid chromatography (HPLC) and Real-time PCR, respectively.

Results: Resistance training increased the content of S1P in plasma (P=0.001) and changed the gene expression of S1P1, S1P2 and S1P3 receptors. There were significant correlations between plasma S1P and gene expression of S1P2, 3 receptors with gene expression of MyoD.

Conclusion: S1P as a growth mediator may play an important role in skeletal muscle adaptations.

Keywords: MyoD, Myogenin; S1P; S1P receptor; Skeletal muscle

Introduction
Skeletal muscle has a high degree of metabolic, structural, contractile and functional plasticity (1). Several growth factors have been shown to affect satellite cells activation and proliferation (2). It has been shown that MyoD and myogenin, as members of myogenic regulatory factors (MRFs) family (3), are myogenic markers of satellite cells activation and are believed to be involved in satellite stem cells proliferation and differentiation, respectively (4). Sphingolipids (sphingosine, sphingosine-1-phosphate (S1P), sphinganine, sphingosine-1-phosphocholine (S1PCh), ceramide and ceramid-1-phosphate) (5) are involved in regulation of proliferation, differentiation, hypertrophy and apoptosis of cells (5, 6). S1P is a platelet-derived sphingolipid (7, 8) that acts as an extracellular mediator through (9) G protein–coupled receptors family (10) S1P1 (EDG-1), S1P2 (EDG-5), S1P3 (EDG-3), S1P4 (EDG-6), and S1P5 (EDG-8) (9). S1P receptors couple to different heterotrimeric G proteins including Gi, q, 12/13 (11). S1P can act through the activation of phospholipase C (PLC), Ca2+ mobilization, activation of extracellular signal-regulated kinases 1/2 (ERK1/2), mitogen-activated protein kinase (MAPK), adenylate cyclase (AC), phosphoinositide 3-kinase (PI3K), small GTPases Rac and Rho, protein kinases Akt, c-Jun N-terminal kinase (JNK), phospholipase D (PLD), and inducing cellular calcium flux and inhibiting cAMP accumulation. S1P1, 2, 3 receptors levels of skeletal muscle are high, among which S1P1 expresses at the highest level (12). Interestingly, following denervation, infusion of S1P and sphingosine increased myoD and myogenin (12). Nagata showed that S1P has an important role in muscular regeneration through regulation of skeletal muscle satellite cells (13, 14). Kim et al. strongly suggest that S1P can affect the

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chondrocyte proliferation (15).

S1P can act as a myogenic differentiation factor through P38MAPK pathway (16). Following injury in skeletal muscle cells SK1 enzyme was activated and caused the increase of levels of endogenous S1P that associated to satellite cells proliferation and differentiation (17). Donati et al. demonstrated the role of S1P in proliferation and survival of mesoangioblast and believed that S1P-dependent signaling pathways may be involved in improvement of muscle repair and regeneration (18). Furthermore, Danielli-Betto et al showed that exogenous S1P stimulates the process of myofibers regeneration. Recently Lou et al illustrated that in dystrophic muscles S1P enhances satellite cell activation through a S1PR2/STAT3 Signaling Pathway. S1P influences epigenetic reprogramming in regenerating muscle (19).

These modifications suggest that S1P signaling is involved in the molecular events which control the early stages of injured muscles repair. In the same line some studies have indicated the bioactive lipid S1P as a novel myogenic factor and have implied to the involvement of sphingolipid pathways in muscle growth (20). Danielli-Betto et al showed that prolonged exercise increases the content of S1P in the soleus and the red gastrocnemius, so they expressed that S1P could be a factor to delay muscle fatigue during long-term exercise (21). To the best of our knowledge, there is no evidence on relation between myogenic markers of satellite cell activation (MyoD and myogenin) and plasma S1P and gene expression of its receptors. Considering the importance of load training as a stimulating model of muscle adaptation, this research aimed at studying possible relations between myogenic regulatory factors and S1P system in skeletal muscles of rat.

Materials and Methods

Animals and Protocol training

Twenty four 8-week-old male Wistar rats (190–250 gr) were obtained from Pasteure institute (Tehran, Iran). All animals were maintained in pairs in a temperature and humidity controlled Room at 22 °C under a 12:12-h light-dark cycle and were allowed normal cage activity. They were fed with standard rat diet and water was also available. After a week of acclimation to the animal facility, the rats were randomly divided into a control group (n=12) and a training group (n=12). The control group remained in cages during the experimental period. Body weight of both groups were monitored weekly and served as a general indicator of the health of the animals. Resistance training ladder was one meter height with 2 cm grid and 85 degree incline (22). After one week familiarizing with resistance training, exercise started using weights attached to the upper portion of base of rat’s tail. The second week began with a load 50% of their individual body mass (23). The load was then increased by 200% of their body mass at the end of 8th week.

A successful repetition was when the animal climbed from the bottom of the rack to the top within 8 seconds. The rats were placed at the bottom of the ladder and motivated to climb the resistance ladder. The only encouragement during the training period was an occasional touch of the base of the animal’s tail (24). The number of climbs (repetitions) performed was 20 repetitions per session. After finishing one set (5 repetitions) rest was permitted for 2 minutes. The animals performed each 5 repetitions of each set with 15s rest between each repetition. The rats trained 3 days/week for 8 weeks. A warm-up program consisting of two sets of five repetitions (with no weight), with a 3 minutes rest between sets was performed by animals. At the end of each session, the rats completed a cool-down consisting of five repetitions (with no weight), with 3 minutes rest between sets.

Measurements

The content of S1P was determined by high pressure liquid chromatography (HPLC) with a fluorescence detector and C18 reversed-phase column system (Aligent 1200 series. NanoLC) (25). S1P and C17-S1P (a 17 carbon analog of S1P as internal standard) were obtained from Avanti Polar Lipids (Alabaster, AL). O-phthalaldehyde (OPA) (suitable for HPLC fluorimetric detection) and Alkaline Phosphatase were obtained from Sigma-Aldrich. Other solutions such as HPLC-grade water, acetonitrile, β-Mercaptoethanol/2-Mercaptoethanol (2ME), ethanol and Methanol were purchased from Merck (Homburg, Germany) (25).

All surgical procedures were done in a single session. The rats were anesthetized by an intraperitoneal injection of Ketamine (75 mg/kg) and Xylazine (20mg/kg) after the 8 weeks of resistance training and 48 h after the last exercise bout to avoid the acute effects of training. The soleus muscle as a slow twitch muscle was removed and studied. The blood samples were spun down in a centrifuge for 15 min at 4 °C temperature and 8000g to remove the blood cells. Plasma and muscles were frozen in liquid nitrogen and stored in -80 °C for later analysis. Total RNA was isolated from ~50 mg muscles of soleus that were powdered under liquid nitrogen using a pestle. Frozen powder was incubated in 1ml Trizol reagent. Then The RNA samples were stored at -80 °C until later analysis. 2µg of total RNA was used to synthesize cDNA using a Kit (Fermentase). Determination of relative mRNA expression was performed by real-time RT-PCR using a Real-Time
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PCR Detection System (Applied Biosystems StepOnePlus™ Real-Time PCR System (48 well)).

Table 1. The weight before and after training, and amount of plasma S1P content.

| Groups                  | Exercise Group (N = 12) | Control Group (N = 12) | t    | P - value |
|-------------------------|-------------------------|------------------------|------|-----------|
| Pre-training weight (gr)| 223.25±11.95            | 224.41±15.77           | 0.204| 0.840     |
| Post-training weight (gr)| 285.83±18.50            | 280.58±16.20           | 0.739| 0.467     |
| Plasma S1P (pmol/ml)    | 1016.38±461.01          | 430.43±195.39          | 4.054| 0.001     **|

The forward (F) and reverse (R) primer sequences for 18S, S1P1, S1P2, S1P3 were as following: 18S - F: GTTGGTTTTTCGGAACTGAGGC, R: GTCCGATCGTTATGGTGCTG (204bp), S1P1 (NM-017301) - F: TCATCCTCGGCAATTCGAAT, R: GAGTGA GCTTGTAGGTTGGTG (273bp), S1P2 (NM-017192) -F: CGGAAGGCACTGACTAATACAGATT, R: TC CCAGCCTCAAGGACACAGT (278bp), S1P3 (XM-225216) -F: ACGCCGCGTTCCTACTCTCTC, R: TGGATCTCTCCAGGATGTTCTG (69bp), MyoD (NM-176079) -F: ACTACACGGCGCGACTCAGAC, R: GTGAAGATGCGCTCCACTAT (208bp), myogenin (NM-017115) -F: TGGTCCCAACCATGAGATCTC, R: AATATCCCTCCACCGTGA (233bp).

The total volume of the reaction tube was 20 µl; 10 µl SYBR Green Master Mix (primer Design, UK), 1.4 µl forward and reverse primers, 5 µl nuclease-free water and 3.6 µL cDNA template. The amplification profile included a denaturation step at 95°C for 15 s, primer annealing at 60°C for 15 s and 30 s at 72°C with 40 cycles (3 steps), and extension at 72°C for 60 s. All samples were run in duplicate. Relative fold changes in mRNA were determined by the delta–delta cycle threshold method after normalizing to the internal control gene. 18s rRNA was used as a control to normalize the mRNA content of the target genes in each sample (26).

Statistical analysis

The findings were analyzed using Pearson correlation and independent t-tests. The results are presented as mean ± standard deviation.

Results

The data showed no significant difference in initial and post training weight between experimental (p = 0.840) and control (p = 0.467) groups. Resistance exercise training increased the total content of S1P in plasma (P = 0.001) in comparison with control group (Table 1). No significant correlation was found between gene expression of S1P1 receptor and MyoD (r = +0.274, p = 0.59), while there were significant correlations between gene expression of S1P2 receptor (r = +0.894, p < 0.000) and S1P3 receptor (r = +0.287, p = 0.048) (Figure 1a).

Figure 1. Correlation between MyoD and S1P receptors (S1P1, S1P2 and S1P3). (A) There is significant correlation between gene expression of MyoD and S1P2 receptor (r = +0.894, p = 0.000). (B) There is significant correlation between gene expression of MyoD and S1P3 receptor (r = +0.274, p = 0.059) (P<0.05) (Figure 1a).

Correlation between Myogenin and S1P receptors (S1P1, S1P2 and S1P3). (A) There is significant correlation between gene expression of Myogenin and S1P1 receptor (r = +0.804, p = 0.000). (B) There is significant correlation between gene expression of MyoD and S1P2 receptor (r = +0.544, p = 0.000). (C) There is significant correlation between gene expression of Myogenin and S1P3 receptor (r = +0.378, p = 0.000) (P<0.05) (Figure 1b).

Correlation between Myogenic regulatory factors (MRFs) and S1P plasma level. (A) There is significant correlation between S1P plasma level and gene expression of Myod (r = +0.554, p = 0.000) and (B) gene expression myogenin (r = +0.315, p = 0.029) (P<0.05) (Figure 1c).

Significant correlation was seen between gene expression of S1P1 receptor and myogenin (r =

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There were significant correlations between plasma S1P levels and gene expressions of MyoD (r=0.544, p < 0.000) and myogenin (r=0.315, p=0.029) (Figure 1c).

Discussion
The results showed significant correlations between plasma S1P and genes expressions of MyoD and myogenin. Some researchers assume S1P as a trophic factor, as this factor can increase gene expression of MyoD and myogenin (r=0.378, p=0.008). Also, significant correlation between gene expression of S1P2 receptor and myogenin (r=0.804, p < 0.000). There was significant correlation between gene expression of S1P3 receptor and myogenin (r=0.544, p < 0.000) was observed (Figure 1b).

There were significant correlations between plasma S1P level and gene expressions of MyoD (r=0.554, p < 0.000) and myogenin (r=0.315, p=0.029) (Figure 1c). 1c).

A noteworthy result of the current study was that myogenic regulatory factors (MRFs) (3), and markers of satellite cell activation. It should be noted that plasma S1P is sufficient to fully occupy the majority of S1PRs (18). So, it could be speculated that S1P receptors are more important in myogenic signaling than plasma S1P level itself. Our results demonstrate that S1P receptors are expressed at skeletal muscle and they seemed to be effective candidates in mediating the growth effects of S1P after resistance training. The present work demonstrates, for the first time, findings on gene expression of MyoD, myogenin and S1P1, 2, 3 receptors in fast (FHL) and slow (SOL) twitch skeletal muscles and S1P content in plasma after 8 weeks resistance training in male Wistar rats. The results clearly show that resistance exercise training markedly affects the S1P levels in plasma. To the best of our knowledge no data in the literature are available on resistance training to be compared with these results, however few studies in which acute and prolonged endurance exercises are used might help. Blachnio-Zabielska et al. showed that acute endurance training increases the content of S1P in the soleus and the red gastrocnemius (33). It has been shown that insulin, cytokines and growth factors, such as PDGF, EGF, IGF-1 activate SK1 acutely, resulting in transient increase of S1P (15, 34, 35). Trenerry et al. (2011) illustrated that after resistance training protein expression of PDGF-BB increased in skeletal muscle (36). Prolonged resistance training increased resting IGF-1 concentrations (38-40). It seems that increased platelet activity and/or platelet count following eight weeks of resistance training caused the increase of S1P levels in plasma (41, 42). Ahmadizad et al. indicated significant increase in platelet count in response to resistance training (43). S1P is likely to be such mediator because it is released from activated platelets and leads to mitogenic effects (44). All these might be due to the resistance training.

In support of our research findings, de la Garza-Rodea et al. showed that S1P/SPL/S1P-receptor axis plays major roles in expression of a number of miRNAs and myogenic differentiation in cell line (45). In another study, Sassoli et al. illustrated that SK1 activity and endogenous S1P levels were significantly higher in the injured fibers which seemed to be associated to satellite stem cells (46). Together, these findings indicate an important role for S1P in regeneration of skeletal muscle and its adaptation to resistance training. Danieli-Betto et al. demonstrated that S1P may be released to the sites of tissue injury and stimulate cellular repair responses (20). Furthermore, resistance training increased gene expression of S1P1, S1P2 and S1P3 of FHL and SOL.

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in experimental group compared to that in control group. Zanin et al. have shown that the gene expression of S1P1, 3 receptors significantly decreased during muscle denervation (12). As denervation causes inactivity and this might lead to decrease in gene expression of S1P receptors, it might be reasonable that resistance training which activates muscles has increased gene expression of these receptors as shown in this study. Resistance training significantly increased gene expression of MyoD in trained fast twitch FHL and slow twitch soleus muscles compared to that of the control group. Furthermore, 8 weeks resistance training significantly increased gene expression of myogenin in trained FHL and SOL compared with control group. MyoD is an essential factor for proliferation whereas myogenin is an important factor in myogenic differentiation (47).

We have shown correlations between satellite cells activation markers (MyoD and myogenin) and plasma S1P content and its receptors before and after resistance training which might strengthen the trophic/myogenic role of S1P system in skeletal muscle.

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Author contributions
All authors have made substantial contributions to the conception and design of the study, doing the experiments, acquisition or statistical analysis and interpretation of data. All authors also contributed in final approval of the version of manuscript.

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Conflict of Interest
There is no conflict for present study.

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