Satellite cells depletion in exercising human skeletal muscle is restored by ginseng component Rg1 supplementation

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ARTICLE INFO

Keywords:
Antioxidant
Inflammation
Myogenisis
Stem cell
Cycling

A B S T R A C T

Rg1 is a steroidal component in Panax ginseng, which increases high intensity exercise performance in humans. Here, we examined the effects of Rg1 supplementation on the dynamical changes of human muscle satellite cells following exercise. Twelve young men ingested Rg1 (5 mg) or Placebo (PLA) 1 h before exercise in a double-blind, placebo-controlled crossover design. Biopsy muscles were collected before, immediately after, and 3 h post-exercise. TNF-α mRNA increased and satellite cell number decreased immediately after exercise, followed by decreases in centrally nucleated myofibers and total glutathione 3 h post-exercise. Rg1 supplementation increased Myo5 mRNA and restored satellite cell number after exercise, and total glutathione and centrally nucleated myofibers 3 h post exercise. Rg1 had no significant effect on TNF-α mRNA response against exercise. The transient exercise response in suppressed centronucleation and satellite cell depletion revered by Rg1 are not associated with TNF-α expression.

1. Introduction

Panax ginseng is a widely used ergogenic supplement, which is generally delivered in form of food, drink and powder. However, its metabolic actions have been confounded by inconsistent component profiles due to changes in harvesting season, soil, and species (Sievenpiper, Arnason, Leiter, & Vuksan, 2003, 2004), representing a major barrier in formulating effective nutraceuticals. Rg1 is a major steroidal component of Panax ginseng, which has been shown to increase endurance performance in humans (Hou et al., 2015; Wu et al., 2018).

Endurance exercise is entropic in nature, which inevitably increases micro-damage of recruited skeletal muscle at high intensity (Ebbeling & Clarkson, 1989; Neubauer, Reichhold, Nernesyan, König, & Wagner, 2008). Nevertheless, such challenge induces a fast muscle remodeling, providing an opportunity for tissue repair and regeneration (Yang et al., 2018). Muscle remodeling involves satellite cell activation, proliferation, differentiation, and nucleus fusion into existing myofiber after physical challenges (Yin, Price, & Rudlicki, 2013). Remodeling myofibers can be recognized by nuclei located in the center of cytoplasm (centronucleation), which is more stress resistant (Narita & Yorifuji, 1999). Satellite cells surrounding damaged myofibers contribute nuclei for myofiber remodeling (Reimann et al., 2004; Seale et al., 2000; Snijders et al., 2015). Satellite cell availability determines the resilience of muscle against physical challenges, which is evidenced by delays in post-injury remodeling and resolution of muscle inflammation in Pax7 + satellite cell-depleted muscle (Sambasivan et al., 2011). Satellite cell number increases in human skeletal muscle 24 h following resistance exercise (Murach et al., 2016; Walker et al., 2012). Immediate response of satellite cell number in human skeletal muscle following endurance exercise remains unclear.

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https://doi.org/10.1016/j.jff.2019.04.032
Received 27 January 2019; Received in revised form 4 April 2019; Accepted 18 April 2019
Available online xxx
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Rg1 supplementation is known to suppress TNF-α expression of human muscle cells in vitro (Go et al., 2017; Ma et al., 2006). Endurance exercise increases TNF-α mRNA of human skeletal muscle (Hou et al., 2015; Louis, Raue, Yang, Jemiolo, & Trappe, 2007). TNF-α functions to stimulate progenitor cell proliferation while it inhibits the differentiation during myogenesis in injured muscle (Li, 2003). Myogenesis requires an induction of the myogenic gene Myf5, which is involved with activation of satellite cells from their progenitors (Montarras et al., 1991; Ott, Bober, Lyons, Arnold, & Buckingham, 1991). Myf5 mRNA decreases rapidly as proliferation switches to differentiation during the late stage of myogenesis (Lindon, Montarras, & Pinset, 1998; Montarras et al., 1991).

In this study, we examined the dynamical changes of satellite cell number, Myf5 expression, and muscle remodeling of skeletal muscle in response to an acute bout of endurance cycling exercise at 70% of maximal oxygen uptake (VO\textsubscript{2max}). To elucidate the potential role of TNF-α expression on exercise-induced responses in satellite cell number, the same experiment was repeated under two conditions: Rg1 supplementation and PLA 1 h before an exercise challenge.

2. Materials and methods

2.1. Participants

Twelve healthy male adults (age 21 ± 0.2 years, height 171 ± 2.2 cm, body mass 65 ± 3.7 kg; VO\textsubscript{2max} 48 ± 1.1 ml kg\textsuperscript{-1} min\textsuperscript{-1}) volunteered to participate in this study. This study was approved by Institutional Review Board of University of Taipei (IRB-2015-004) and conformed to the Declaration of Helsinki, as a part of study published elsewhere (Hou et al., 2015). All participants were informed of the procedures and potential risks associated with the study and gave their written informed consent before the study.

2.2. Ginsenoside Rg1

Rg1 is a ginseng-based compound obtained from NuLiv Science, Inc. (Brea, CA, USA). The purity was confirmed by high-performance liquid chromatography (HPLC). Chemical structure of Rg1 extract is shown in Fig. 1. A dose of 5 mg of Rg1 was used based on a previous human study which showed a significant improvement in endurance performance (Hou et al., 2015; Wu et al., 2018). The Rg1 amount used is equivalent to ~2.3 g of ginseng.

2.3. Experimental design

All participants visited the laboratory twice to ingest either placebo (PLA) or Rg1 (5 mg), in a double-blind, placebo-controlled cross-over design. The two trials were separated by a two-week washout period, and were preceded by a familiarization session, which involved all testing procedures and equipment. All participants were provided with a standard isocaloric diet 12 h prior to each trial to avoid potential dietary effect on the outcome variables. A baseline VO\textsubscript{2max} test using an incremental exercise ramp protocol was performed, on a cycle ergometer (Monark 839E, Stockholm, Sweden), one week prior to performing the experimental trials. Rg1 or PLA was orally ingested 1 h before performing a 1 h continuous cycling exercise at 70% VO\textsubscript{2max}. After exercise (within 10 min), participants consumed a high carbohydrate meal (1.5 g carbohydrates per kg body weight, 80% carbohydrate, 8% fat and 12% protein, GI: 80) for recovery, and they were allowed to drink additional water ad libitum.

2.4. Blood sample analysis

Blood samples were collected before and after exercise via a 20-G polyethylene catheter (Jelco, Tampa, FL, USA) placed in an antecubital vein. During recovery, blood samples were collected every 30 min for 180 min. Blood glucose was determined by an automated glucose analyzer (YSI Life Sciences, Yellow Springs, OH, USA). Serum insulin concentrations were measured by using the enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Mercodia, Uppsala, Sweden) according to the manufacturer’s instruction.

2.5. Muscle biopsy

The entire procedure is detailed in a previous study (Wu et al., 2018). Muscle samples were taken from vastus lateralis muscle before (Pre), immediately (0h) and 3h after exercise, under local anesthesia (2% lidocaine) using a 18G Temno disposable cutting needle (Cardinal Health, Waukegan, IL, USA) by a certified physician. Biopsies were taken in vastus lateralis positioned at 3 cm depth, 20 cm proximal to knee. Baseline muscle biopsy (Pre) in the vastus lateralis was conducted 4 weeks before exercise challenge to prevent potential interference of biopsy on exercise-induced muscle inflammation and satellite cell change. Two consecutive muscle biopsies were performed immediately (0h) after and 3 h after endurance cycling at 70% VO\textsubscript{2max}. Muscle biopsies were taken from contralateral leg at the same position on the second and third biopsies. Muscle samples were frozen directly in liquid nitrogen before analysis. Muscle samples for immunohistochemical analysis was disposed immediately into a conical vial containing 10% formalin. Paraffin-embedded tissue was sectioned no later than 3 h following muscle sample collection.

2.6. Glycogen assay

Approximately 15 mg of skeletal muscle from vastus lateralis was dissolved in 500 μl KOH (1N) at 75 °C for 30 min. Dissolved homogenate was neutralized by glacial acetic acid and incubated overnight in acetate buffer (0.3 M sodium acetate, pH = 4.8) containing amyloglucosidase (Roche Diagnostics, Indianapolis, IN, USA). The reaction mixture was neutralized with NaOH (1N). Then all samples were analyzed by measuring glucosyl units by the Trinder reaction (Sigma, St. Louis, MO, USA).
Fig. 2. Glycogen, glucose and insulin. Similar glycogen depletion in vastus lateralis muscle in both PLA and Rg1 trials following high intensity (70%VO2max) cycling exercise (A). Similar response of serum glucose (B) and insulin (C) after meal in both trials during the 3h recovery. Values are presented as means ± SE (N = 12). *Significantly different from baseline (Pre), P < 0.05. PLA: Placebo.

2.7. Glutathione assay

Oxidized (GSSG) and reduced (GSH) glutathione were quantified by a DTNB-glutathione reductase recycling assay, according to an instruction of Glutathione Assay Kit (#703002) (Cayman, Ann Arbor, MI, USA). Briefly, approximately 15 mg muscle samples were homogenized in cold buffer (50 MES, 1 mM EDTA) at the ratio 1:50 (w/v). Homogenate samples (70 μl) were added to 10% metaphosphoric acid and centrifuged at 2000 g for 5 min. Supernatant (60 μl) was mixed with 3 μl triethanolamine (4 M solution). Deproteination sample (50 μl) was used to perform colorimetric detection by the technique of end-point method with absorbance at 405 nm.

2.8. RNA analysis

RNA was extracted from approximately 15 mg of skeletal muscle using TRI Reagent (T9424-200) (Sigma, St. Louis, MO, USA) for homogenization, followed by isopropanol precipitation, two ethanol washes, drying, and suspension in 20 μl nuclease-free water. One microgram of RNA in a total volume of 20 μl was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (#170-8890) (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed using MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), TaqMan Probe (Sigma-Aldrich, Singapore) and iQ Supermix kit (#170-8860) (Bio-Rad, Hercules, CA, USA). The PCR conditions for all genes consisted of one
2.9. Histochemistry and immunohistochemistry

For histological analysis, we identified centrally nucleated muscle fibers on cross sections with hematoxylin and eosin (H&E) staining. Remodeling fibers were identified by centrally located nuclei. We calculated the percentage of remodeling fibers from the whole cross section fibers. The sections were observed under a light microscope (OLYMPUS BX51, Olympus Corporation, Tokyo, Japan), and digital images were taken covering the entire cross section of the vastus lateralis. Satellite cells were also identified by an antibody against Pax7 (Millipore MAB-2100, Darmstadt, Germany). Briefly, sections were fixed using 4% paraformaldehyde for 5 min at room temperature followed by three 5 min rinses with PBS. Sections were blocked with 10% goat serum plus 0.5% Triton-X 100 for 45 min. Sections were incubate overnight at 4°C with primary antibody (mouse anti-Pax7, 1:100). The staining resulted in nuclei staining blue and Pax7 + cells identified as any nuclei localized to the membrane (basal lamina) of a muscle fiber and stained brown. The sections were observed under a light microscope (Olympus BX51, Olympus Corporation, TKY, Japan), and digital images were taken covering the entire cross section of the vastus lateralis. The slides were reviewed at a magnification of ×200 and ×400 by a certified pathologist. Optical images were analysis using ImageJ (NIH). Cell positive markers were quantified and expressed as positive signal number/total fibers (%).

2.10. Statistical analysis

All data are expressed as means ± SE. The data were analyzed using a two-factor repeated-measures ANOVA, with Rg1 supplement as the within-factor and three time points as between-factor (before exercise, 0h after exercise, and 3h after exercise). Post hoc paired comparison used Fisher LSD analysis. Area under the curve (AUC) was calculated using trapezoidal method, and was compared between the treatments using a paired t-test. Type I error of P < 0.05 is considered significant and P = 0.05–0.10 is considered moderately significant.

3. Results

3.1. Glycogen depletion after exercise

This 1-h cycling exercise effectively depleted muscle glycogen of participants under both PLA and Rg1-supplemented conditions to a similar extent (~31% and ~36%, ANOVA time effect P < 0.001) (Fig. 2A). During a 3-h recovery with carbohydrate ingestion, the amount of glycogen replenishment, areas under curve (AUC) of glucose (Fig. 2B) and AUC of insulin (Fig. 2C) were also similar for the PLA and Rg1-supplemented conditions.

3.2. Increased skeletal muscle TNF-α mRNA after exercise

TNF-α mRNA levels in vastus lateralis muscle increased after exercise in both PLA (0h: +3.6 fold, P < 0.05; 3h: +4.0 fold, P < 0.05) and Rg1 trial (0h: +3.7 fold, P = 0.09; 3h: +2.5 fold, P < 0.05) (Fig. 3A).
3.3. Decreased glutathione after exercise was attenuated by Rg1 supplementation

Total glutathione in skeletal muscle was significantly reduced at 3 h post exercise in PLA trial and this depletion was absent in the Rg1 trial (Fig. 3B, P < 0.05). The GSH/GSSG ratio was unchanged after exercise (Fig. 3C) and no supplement difference was found between two trials.

3.4. Accelerated repletion of satellite cells after exercise by Rg1 supplementation

Pax7+ satellite cell number in vastus lateralis was assessed by immunohistochemistry staining before (Pre), immediately (0 h) and 3 h after 1 h cycling at 70% VO2max. The quantity of Pax7 positive cells per fiber is similar to previous report (Christov et al., 2007). Pax7+ satellite cells transiently declined by 36% below baseline level (P < 0.05), and rapidly recovered 3 h after exercise (Fig. 4A). Centrally nucleated fiber of the muscle decreased by 46%, 3 h after exercise (P = 0.08, Fig. 4B). These changes were completely prevented by Rg1 supplementation (P < 0.05).

3.5. Transient myogenic induction by Rg1 supplementation

Increased expression levels of Myf5 mRNA and MRF4 mRNA are hallmarks of myogenic induction (Lindon et al., 1998). No changes in Myf5 mRNA and MRF4 mRNA expressions of muscle were observed after exercise during PLA trial (Fig. 5). Transient increase ( +81%, P < 0.05) in Myf5 mRNA level of muscle was found immediately after exercise during the Rg1 trial, compared against PLA trial. Similar trend was also found for MRF4 mRNA expression ( +86%, P = 0.09).

4. Discussion

Increased satellite cell number in exercised skeletal muscles were often measured long after resistance exercise (Snijders et al., 2015; Walker et al., 2012). In this study, we further examined dynamical changes against aerobic exercise challenge. Our data show, for the first time, a transient depletion of satellite cells occurred immediately after exercise, concurrent with increases in TNF-α mRNA in human skeletal muscle. This is followed by a reduced percentage of centrally nucleated myoblasts and glutathione depletion 3 h post-exercise. Satellite cells were quickly restored following a 3-h recovery, which suggests that satellite cell replenishment in skeletal muscle can occur in a rapid pace. Here we also found that the exercise-induced depletion of satellite cells can be attenuated when Rg1 is supplemented 1 h before exercise.

In this study, one key difference between PLA and Rg1 trials is the response of myogenic determinant expression (Myf5 mRNA and MRF4 mRNA) following exercise. In particular, myogenic gene expression in exercising skeletal muscle is increased by >80% in the Rg1 trial. Induction of Myf5 mRNA expression is generally regarded as a hallmark for commitment of myogenesis (Weintraub et al., 1991). In the absence of Rg1, we found no change in Myf5 mRNA within the exercising muscle, which is consistent with previous study using a slightly lower exercise intensity (Yang, Creer, Jemiolo, & Trappe, 2005). Interestingly, we also found that during the Rg1 trial, the increased Myf5 expression in exercising muscle was associated with attenuated Pax7+ satellite cell depletion, and subsequently preservation of centrally nucleated myoblasts. Accelerated myogenesis of exercising skeletal muscle may also explain improved endurance performance after Rg1 supplementation reported previously (Hou et al., 2015). It is likely that Rg1 enhances fa-
Fig. 5. Myf5 mRNA and MRF4 mRNA in exercising human skeletal muscle. Myf5 mRNA transiently increased after exercise by Rg1 supplementation (A). MRF4 mRNA shows similar trend (P = 0.09) (B). Expression levels are normalized to baseline (Pre). Values are expressed as means ± SE (N = 12). *Significantly different from PLA, P < 0.05. PLA: Placebo.

5. Conclusions

Exercise-induced muscle TNF-α mRNA expression between trials observed suggests that stabilization of satellite cell number by Rg1 may be unrelated to TNF-α expression.

The number of centronucleation is a marker of regenerating myofiber (Narita & Yorifuji, 1999), which is decreased 3h after exercise, and can be reserved by Rg1 supplementation before exercise (Fig. 4). Decreased number of centrally nucleated fiber 3h after exercise occurred after satellite cell depletion implicating that an acute shortage of nucleus source from satellite cells for muscle regeneration can occur during intensive muscle contraction. The main limitation of this study is that satellite cells are not the only source of nuclei for myofiber renewal in challenged muscle tissue (Zammit, Partridge, & Yablonska Reuveni, 2006) and it remains unclear whether other stem cell types (e.g. bone marrow cells or other intramuscular cells) contribute to nucleus donation.

Ethics statements

This study was approved by Institutional Review Board of University of Taipei (IRB-2015-004) and conformed to the Declaration of Helsinki. All participants were informed of the procedures and potential risks associated with the study and gave their written informed consent before the study.

Conflict of interest

This work was funded for a purpose to produce an ergogenic supplement Actigyn for Nuliv Science, USA. JFW and CHK are listed as an inventor on a patent application in US submitted.

Acknowledgements

This study was supported by Nuliv Science Taiwan, Ministry of Science & Technology Taiwan (grant number: 106-2410-H-845-019, 107-2410-H-845-019), and University of Taipei, Taiwan. The authors wish to thank professional pathologists from the Taipei Pathology Institute and Changhua Christian Hospital for invaluable technical assistance.

References


tigue recovery by accelerating myogenesis in exercising skeletal muscle.

Glutathione is essential to myogenesis. Here, we observed ~40% glutathione depletion in skeletal muscle 3h after cycling in the PLA trial in consistency with a previous study (Medved et al., 2004). The reduced centrally nucleated myofibers 3h after exercise could be associated with this brief glutathione depletion. Glutathione is required for development of myotubes from satellite cells during myogenesis, evidenced by a blunted myogenic differentiation of murine skeletal muscle C2C12 myoblasts after glutathione depletion (Ardite, Barbera, Rocca, & Fernández Checa, 2004). Despite that glutathione depletion was attenuated by Rg1, the differences between PLA and Rg1 were too small to infer a significant effect on normalizing centronucleation (Figs. 3B and 4B).

In this study, stabilization of satellite cell number in exercising skeletal muscle by Rg1 supplementation is unlikely to be associated with TNF-α expression. Previous in vitro studies have shown that Rg1 administration can inhibit TNF-α expression in human muscle cells (Go et al., 2017; Ma et al., 2006), but no studies have been done in vivo. In vitro, TNF-α inhibits myogenesis by decreasing myotube number and myogenic fusion (Trendelenburg, Meyer, Jacobi, Feige, & Glass, 2012). This is also supported by evidence of increased muscle satellite cell number and Pax7 expression following anti-TNF-α treatment in mdx mice (Palacios et al., 2010). TNF-α appears to delay myogenesis by keeping satellite cells in the early, proliferative stages of myogenesis (Tidball, Dorshkind, & Wehling Henricks, 2014). Prior to this study, it was unclear whether the satellite cell number stabilization of exercising human skeletal muscle was mediated via suppressing TNF-α expression by Rg1 supplementation. However, in this study, the lack of difference


