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Cannabinoid receptor 1 and acute resistance exercise – *In vivo* and *in vitro* studies in human skeletal muscle

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ABSTRACT

Aim: This study aimed to determine whether Cannabinoid receptor 1 (CB1) is involved in mammalian target of rapamycin (mTOR) signaling and skeletal muscle protein synthesis.
Methods: This study used human *vastus lateralis* skeletal muscle biopsies obtained before and after a resistance exercise (RE) bout in young men ($n = 18$). The signaling mechanisms were studied *in vitro* in human myotubes. Protein expression was determined by Western blot and confocal microscopy, and gene expression by quantitative PCR. Protein synthesis was measured *in vitro* using puromycin-based SuNSET technique.
Results: In human skeletal muscle, an anabolic stimulus in the form of RE down-regulated CB1 expression. The negative change in CB1 expression was associated with increased phosphorylation of mTOR signaling proteins. *In vitro*, CB1 antagonist AM251 induced phosphorylation of mTOR downstream targets, ribosomal protein S6 kinase (S6K1), S6 and eukaryotic initiation factor 4E binding protein (4E-BP1) in human myotubes. These effects were ERK1/2-dependent and insensitive to mTOR inhibitor, rapamycin. Compared to AM251 treatment alone, inhibition of ERK1/2 by UO126 in the presence of AM251 decreased phosphorylation of S6K1, S6 and and 4E-BP1 at $Thr^{37/46}$. AM251 increased protein synthesis in cultured human myotubes, which was not rapamycin-sensitive but was ERK1/2-dependent.
Conclusions: Our results indicate that RE down-regulates CB1 expression. Inhibition of CB1 signaling increases skeletal muscle anabolic signaling down-stream of mTOR and protein synthesis through ERK1/2. Our study may provide base for the development of CB1-blocking drugs to treat or prevent muscle wasting.

Introduction

The endogenous cannabinoid system (ECS) is a ubiquitous lipid-derived signaling system that plays an important role in the onset of cardio-metabolic diseases primarily *via* cannabinoid type 1 receptor (CB1) [1]. In the skeletal muscle, CB1 regulates basal and insulin mediated glucose transport [2], lipid metabolism [3] and oxidative pathways [4]. However, the possible role of CB1 in skeletal muscle protein synthesis remains to be established.

Abbreviations: CB1, cannabinoid receptor 1; mTOR, mechanistic target of rapamycin, but initially mammalian target of rapamycin; RE, resistance exercise; S6K1, ribosomal protein S6 kinase; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; ECS, endogenous cannabinoid system; PLD, Phospholipase D; PA, phosphatidic acid.

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Protein synthesis and translation in the skeletal muscle is stimulated by the mechanistic target of rapamycin (mTOR) pathway [5]. Activation of mTOR increases mRNA translation e.g. *via* its downstream effectors ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) [6]. 4E-BP1 inhibits translation by binding to the translation initiation factor eIF4E, and hyperphosphorylation of 4E-BP1 disrupts this interaction resulting in activation of the cap-dependent translation [7]. Anabolic stimulus and adequate nutrition stimulates the mTOR signaling pathway [8] resulting in increased protein synthesis and muscle hypertrophy [9]. However, the cellular mechanism(s) upstream of contraction-induced activation of mTOR remain controversial [10].

Accumulating evidence indicates that contraction-induced activation of mTOR does not require conventional growth factor signaling [11,12]. Early studies suggested that phospholipase D (PLD) and its membrane derived lipid messenger phosphatidic acid (PA) are involved in RE-induced activation of mTOR [13,14], but later on it has been shown that in addition to PLD, diacylglycerol kinase ζ plays an important role in mechanically induced increases in PA and mTOR signaling [15]. These findings suggest that locally-derived membrane receptor-based signals may represent one of the mechanisms by which muscle adapts to RE [10]. Given the nature of ECS as an intrinsic local lipid-derived signaling system, and the fact that PA and anandamide (AEA, an endogenous ligand of CB1 receptor) share the same biosynthetic pathway [16], it is possible that mTOR pathway and CB1 are interconnected. Accordingly, it has been shown that in brain CB1 increases mTOR signaling [17].

In addition to mTOR pathway, exercise can modulate ECS activity. Animal studies have shown that exercise reduces CB1 receptor expression in mice skeletal muscle [18]. In humans, several lines of evidence indicate that the ECS may participate in the adaptive responses to exercise [19]. For instance, acute aerobic exercise increases the plasma levels of AEA [20], which has been further shown to improve insulin sensitivity, glucose uptake and mitochondrial biogenesis in human skeletal muscle cells [21]. However, no human studies have reported the effects of exercise on skeletal muscle CB1 expression.

Therefore, we hypothesized that RE modulates CB1 expression in human skeletal muscle and that the changes in CB1 are associated with the changes in mTOR pathway proteins and further with protein synthesis. To test our hypothesis a series of experiments were conducted. First, we used skeletal muscle samples obtained from 18 males before and after a single heavy RE-bout to study the association between CB1 and mTOR signaling. Further, the effects of CB1 on mTOR-related signaling and protein synthesis were studied *in vitro* in human skeletal muscle cells.

Materials and methods

Human study subjects

The study was approved by the local Ethics Committee and was conducted in accordance with the Declaration of Helsinki. The study subjects consisted of volunteered non-athlete, healthy 18 males aged 20–30 years. The subjects were recruited for the study by advertising in newspapers and through email lists. All gave their informed consent before participation. The subjects were moderately active and their normal habitual activities included walking, jogging, swimming or ball-games. None of the subjects had any regular resistance training experience.

The study was designed as described previously [22]. First, to determine 1 repetition maximum (1RM) David 210 system was used to measure maximal bilateral concentric force production for leg extensors (hip and knee extensors). Separate trials were performed for concentric 1 repetition maximum (RM) testing. After each repetition, the load was increased until the subject was unable to extend his legs from ~60° to the full-extended ~180° knee angle position. The highest successful load was determined as the 1 RM. The subjects were carefully familiarized with the test procedures and had several warm-up contractions in all devices before the actual testing.

Then in a separate day, a bilateral leg press machine (David 210, David Fitness and Medical) was used for the single heavy RE bout. The total number of sets was five. Each set contained 10 repetition maximums (RM). Recovery time between the sets was 2 min. The first set started with the 75% 1RM load based on the two earlier strength tests to measure baseline strength of the subjects. The loads were adjusted during the course of the RE bout due to fatigue so that each subject would be able to perform 10 repetitions at each set. The duration of concentric and eccentric contractions were standardized to be both ~2 s since contraction length affects muscle responses. This type of exercise led to muscle hypertrophy when followed for 21-weeks [22].

Muscle biopsies were obtained 0.5 h before (baseline, BL) and 1 h (1 h post-RE) and 48 h (48 h post-RE) after the bout of RE using Bergström needle. The baseline and the 48 h biopsies were taken from the right leg. To avoid any residual effects of the prebiopsy, the 1 h post-RE biopsy was taken from the left leg and the 48 h post-RE biopsy was taken 2 cm above the previous biopsy location.

Dietary intakes of the subjects were registered with dietary diaries 3 days before the biopsy day, on the biopsy day, and the day thereafter (5 days overall). All of the diaries were analysed using the Micro Nutrica nutrient-analysis software version 3.11, developed and maintained by Social Insurance Institution of Finland. The Micro-Nutrica database contains 66 dietary factors, 680 different food items, and about 640 dishes commonly consumed in Finland.

Body fat was measured with skinfolds (biceps and triceps brachii, subscapular and iliac crest).

Cells and reagents

Primary human muscle cell line (CHQ) was derived from quadriceps muscle biopsy of a 5-day-old, healthy infant, in accordance with the current legislation [23]. The cells were maintained as non-differentiated mononuclear myoblasts by cultivation in 5:1 DMEM (Glutamax):199 medium supplemented with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% sodium pyruvate. To differentiate the cells into multinuclear myotubes prior to experiments the cells were maintained for 5 days in DMEM (Glutamax) supplemented with 10 U/mL penicillin, 10 μg/mL streptomycin, 1% sodium pyruvate, and 10 μg/mL insulin. Insulin was purchased from Sigma–Aldrich (St. Louis, USA) and the rest of the media components from Invitrogen (Carlsbad, CA, USA). AM251 and rapamycin were purchased from Cayman Chemical (Ann Arbor, MI, USA). For the cell culture experiments 100 nM rapamycin was used to inhibit mTOR. The CB1 antagonist AM251 exhibits an IC50 for G-protein-coupled receptor activity of 8 nM, and a K_i of 7.5 nM [24]. 100 nM concentration of the antagonist was chosen for the experiments as it has been shown to be effective in cultured skeletal muscle cells [4]. The inhibition of CB1 signaling at 100 nM AM251 was validated by determining the expression of *SCD1* mRNA, which CB1 is known to regulate. *SCD1* expression decreased in response to AM251 in myotubes by over 10-fold (data not shown). Cells were treated with AM251 for 30 min. UO126, which was used to inhibit ERK1/2 in cell culture experiments was used at concentration of 5 μM. UO126 was purchased from Sigma–Aldrich. When the cells were co-treated with AM251 and rapamycin or UO126, the latter reagents were added 30 min and 1 h prior to AM251 in order to inhibit mTOR or ERK1/2, respectively.

Protein synthesis in cultured human myotubes was measured using "SuNSET", a recently developed nonradioactive puromycin-based method [25,26]. This technique uses puromycin (a structural