Published in final edited form as:
J Nutr Biochem. 2014 January ; 25(1): . doi:10.1016/j.jnutbio.2013.09.007.

Effects of (−)-epicatechin on molecular modulators of skeletal muscle growth and differentiation

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Abstract

Sarcopenia is a notable and debilitating age-associated condition. Flavonoids are known for their healthy effects and limited toxicity. The flavanol (−)-epicatechin (Epi) enhances exercise capacity in mice and Epi-rich cocoa improves skeletal muscle structure in heart failure patients. (−)-Epicatechin may thus, hold promise as treatment for sarcopenia.

We examined changes in protein levels of molecular modulators of growth and differentiation in young vs. old, human and mouse skeletal muscle. We report the effects of Epi in mice and the results of an initial proof-of-concept trial in humans, where muscle strength and levels of modulators of muscle growth were measured. In mice, myostatin and senescence-associated β-galactosidase levels increase with aging, while those of follistatin and Myf5 decrease. (−)-Epicatechin decreases myostatin and β-galactosidase and increases levels of markers of muscle growth. In humans, myostatin and β-galactosidase increase with aging while follistatin, MyoD and myogenin decrease. Treatment for 7 days with (−)-epicatechin increases hand grip strength and the ratio of plasma follistatin/myostatin.

In conclusion, aging has deleterious effects on modulators of muscle growth/differentiation, the consumption of modest amounts of the flavanol (−)-epicatechin can partially reverse these changes. This flavanol warrants its comprehensive evaluation for the treatment of sarcopenia

Keywords

Epicatechin; sarcopenia; flavanoids

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Conflict of interest
None to disclose.

Introduction

One of the most notable and debilitating age-associated alterations is the progressive loss of fat-free skeletal muscle (SkM) mass and strength, a condition known as sarcopenia [1]. This loss of muscle mass and strength, and the increase in body fat with aging occur in part as a consequence of metabolic changes associated with an inactive lifestyle [2]. Currently, only exercise is recognized as an effective means to counteract sarcopenia. However, physical activity in the older population is often restricted by a variety of conditions and/or diseases. Reports indicate that SkM strength is inversely associated with all cause of mortality in men [3]. Thus, novel pharmacological strategies that effectively treat sarcopenia can also be seen as a means to reduce frailty in older subjects.

The consumption of modest amounts of cacao products (i.e. cocoa) rich in the flavanol (−)-epicatechin (Epi), has been associated with a significant reduction in cardiometabolic risks [4].

We recently reported on the beneficial effects that the consumption of Epi-rich cocoa had on SkM mitochondria of older patients with heart failure and type 2 diabetes mellitus [5]. We have also demonstrated in 1 year old mice, that treatment with Epi for 2 weeks leads to ~50% increase in exercise capacity that is accompanied by enhanced SkM oxidative capacity and angiogenesis [6,7]. As with exercise, Epi may thus, limit the age-associated decline in SkM structure/function. Interestingly, neither the effects of aging on recognized molecular modulators and/or markers of SkM growth, differentiation and senescence nor the possible effects of Epi on these parameters or muscle strength in humans have been explored. Determining the effects of Epi on these endpoints is thus, key to gauge the potential of the flavanol to be used for the treatment of sarcopenia.

Methods and Materials

Animal studies

We examined the effects of aging on protein levels of recognized modulators of SkM growth (myostatin, follistatin) [8], senescence-associated β-galactosidase (SA-β-Gal) [9] and myogenic differentiation (myogenin, MyoD, MEF2A, Myf5) [10] and report on the effects of Epi treatment in young (6 month old, n=5/group) and old (26 month, n=5/group) C57BL/6 male mice. For these studies, quadriceps muscle samples were obtained from mice treated for 2 weeks with either vehicle by gavage (water) or Epi (1 mg/kg BID) and immediately frozen. Studies in animals were approved by UCSD's IACUC.

Human studies

Frozen, quadriceps SkM samples were obtained from a human biopsy tissue bank and were used to compare the same markers as above, in normal young (28.5±7 years, n=6) vs. old (62±2 years, n=6) subjects. An initial, pilot study was also performed in human subjects (n=6, average age 41±5 years and weight 77.4±7.7 Kg) to assess the effects of Epi treatment on muscle strength and plasma levels of myostatin and follistatin. Plasma myostatin and follistatin levels can change in association with those in muscle in response to, for example, exercise [11,12] and were thus, used as a surrogate for possible responses occurring in SkM. Human subjects were treated for 7 days with 25 mg of pure Epi (Sigma-Aldrich) provided in capsules BID (~1 mg/kg/day). Muscle strength was assessed by hand grip dynamometry (thrice with each hand, alternating hands between trials and resting for 10 seconds in order to prevent fatigue). Maximum strength attained was used for analysis. Blood (plasma) samples were collected before and after treatment and immediately frozen. Studies in human subjects were approved by the Instituto Politecnico Nacional IRB board and conform to all international regulations.

Western blots

Protein levels for the above stated markers were analyzed in SkM or plasma by Western blots and normalized for loading differences using GAPDH. Approximately 20 mg of SkM was homogenized with a polytron in 500 μl lysis buffer (1% Triton X-100, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, and 0.1% SDS) with a protease and phosphatase inhibitor cocktails (P2714 and P2850, Sigma-Aldrich) supplemented with 0.15 mM PMSF, 5 mM Na3VO4 and 3 mM NaF. Homogenates were passed through an insulin syringe five times, sonicated for 30 min at 4°C and centrifuged (12,000 g) for 10 min. The total protein content was measured in the supernatant using the Bradford method. A total of 40 μg of protein was loaded onto a 4–15% precast TGX polyacrylamide gel (Bio-Rad), electrotransferred to polyvinyl membranes, incubated for 1 h in blocking solution (5% non-fat dry milk in TBS plus 0.1% Tween 20 (TBS-T)),followed by a 3 h incubation at room temperature with primary antibodies. Primary antibodies used include myostatin, myogenin, MEF2, MyoD, Myf5 (Abcam), follistatin (Santa Cruz), SA-β- Gal (Millipore) and, GAPDH (Cell Signaling). Primary antibodies were diluted in TBS-T plus 5% non-fat dry milk. Membranes were washed (3 X for 5 min) in TBS-T and incubated 1 h at room temperature in the presence of HRP-conjugated secondary antibodies diluted in blocking solution. Membranes were again washed 3 times in TBS-T, and the immunoblots were developed using an ECL Plus detection kit (Amersham-GE). Western blots of plasma samples (3 μl) were performed to compare the relative levels of myostatin and follistatin (before vs. after Epi). Band intensities were digitally quantified using Image J software (http://www.nih.gov) and values recorded as arbitrary units. All Western blot images were obtained within the linear range of X-ray film to ensure densitometry accuracy.

Statistical analysis

Paired/unpaired t-tests (when appropriate) were used to compare differences between groups.Values reported are mean ± SEM, statistical significance was defined as $P<0.05$.

Results

Animal studies

In mice, aging led to an 18% increase in myostatin ($P=0.06$) and 30% decreases in follistatin and Myf5 protein levels ($P<0.01$, figure 1A, B). An increase of 97% was observed in SA-β-Gal ($P<0.01$, figure 1C). In young and old mice, Epi treatment significantly decreases myostatin levels (15 and 21% respectively), while follistatin increases (56%) in old muscle. Of particular interest is that myostatin levels of treated old mice were similar to those of untreated young animals. Significant increases with treatment were also noted in MEF2 (10%, 19%), Myf5 (12%, 15%) and myogenin (16%, 21%) in young and old samples and MyoD in old SkM (19%). Epi treatment significantly decreased SA-β-Gal in old SkM (22%).

Human studies

In human subjects, with aging, SkM levels of myostatin and SA-β-Gal significantly increase (28%, 48%) while those of follistatin (30%), MyoD (41%) and myogenin (47%) decrease, changes largely in concert with mouse results (figure 2A-C). Treatment for 7 days with Epi yielded a bilateral increase in hand strength of ~7% which was accompanied by a significant increase (49.2 ± 16.6 %) in the ratio of plasma follistatin/myostatin levels (data not shown).

Discussion

The U.S. healthcare cost of sarcopenia was estimated in 2000 at ~$20 billion and is growing as the population ages [13]. In a recent review, current non-exercise based treatment options were discussed including nutritional supplements, hormone therapy (testosterone, estrogens, growth hormone), vitamin D, angiotensin converting enzyme inhibitors and creatine [13]. With the exception of vitamin D, where a positive effect may be observed in those suffering from a deficiency, the other treatments are noted to be either risky or unproven. Three new potential treatments were suggested. Myostatin antagonists, peroxisomeproliferator-activated-receptor-δ agonists (GW1516) and adenosine monophosphate (AMP) activated protein kinase activators such as AICAR. GW1516 was tested in rodents results show stimulated exercise capacity only when used in combination with exercise [13]. AICAR was also reported to enhance exercise capacity in mice by ~44% but has toxic effects [14]. The development of novel and safe therapies is thus, warranted given the typical older population profile of subjects with sarcopenia and likely indefinite period of treatment. Epi is a naturally occurring flavanol found in cacao and green tea and has been orally ingested safely by humans for many centuries. Literature on human studies using high-flavanol cocoa indicate that Epi-rich preparations can be administered safely over extended periods [15]. Furthermore, clinical studies using pure Epi (oral doses of 1-2 mg/kg) have not reported adverse effects [16]. In rats given a green tea extract, the no-observable-adverse-effect level for daily dosing for 6 months corresponded to 85 mg Epi/kg [17], far in excess of the dose used in our published study where we demonstrated increases in exercise capacity of ≈ 50% with 1 mg/Kg BID Epi treatment [6]. Huttermann et al also recently reported that after the withdrawal of Epi (1 mg/Kg BID) animals retain the stimulatory effect on exercise capacity 14 days after treatment whereas exercise conditioned animals failed to do so [18]. Given these facts, Epi appears promising as a treatment for sarcopenia.

As noted above, compounds that inhibit myostatin effects are of interest. Myostatin is recognized as a major inhibitor of muscle growth and its SkM and/or blood levels are reported to increase with aging and in muscle wasting diseases [8]. However, so far, no compounds have demonstrated myostatin inhibitory effects. An alternative means of inhibiting myostatin effects is to increase follistatin, which inhibits myostatin activity by attaching to it and interfering with receptor binding [8]. A molecular signature consistent with stimulatory effects on SkM may be represented by the upregulation of protein levels of modulators of differentiation such as MEF2A, Myf5, MyoD and myogenin [10]. In an attempt to examine the potential of Epi to exert positive effects, we compared the relative levels of the above-referred endpoints in young vs. older cohorts of mice and their responses to treatment. Young and old SkM, respond substantially to Epi. Older mice demonstrated a collection of significant changes in all endpoints suggesting favorable shifts for modulators of SkM growth, differentiation and markers of aging (SA-β-Gal) [9]. In order to define a baseline for future studies using Epi in humans, we characterized the molecular signature of the above noted endpoints in young vs. old SkM. When comparing SkM protein levels of young vs. older cohorts significant increases were noted in myostatin and SA-β-Gal and decreases in follistatin, MyoD and myogenin. On the basis of the promising results derived from the effects of Epi treatment inmice, an initial, proof-of-concept study was implemented in humans. Following 7 days of Epitreatment, significant positive effects were observed in circulating follistatin/myostatin plasma levels and grip strength. Thus, Epi is the first compound ever noted, to favorably modulate both regulators of muscle growth and suggest increases in strength.

These initial study results are provocative and warrant further rigorous examination. An optimal clinical trial design would need to include a significant number of subjects, different age and sex cohorts, use placebo groups and be blinded. Rigorous measures of SkM mass,

strength and endurance would need to be included such as the use of Dual-energy X-ray absorptiometry (DEXA) scans, dynamometry and cycle ergometry. The pursuit of such trials will hopefully shed light on the prospects for an emerging natural and safe compound to be used in the treatment of sarcopenia.

Acknowledgments

Funding. Support was provided to Dr. Ceballos by CONACYT 129889 and to Dr. Villarreal by NIH grants AT4277, P60MD220, HL43617.

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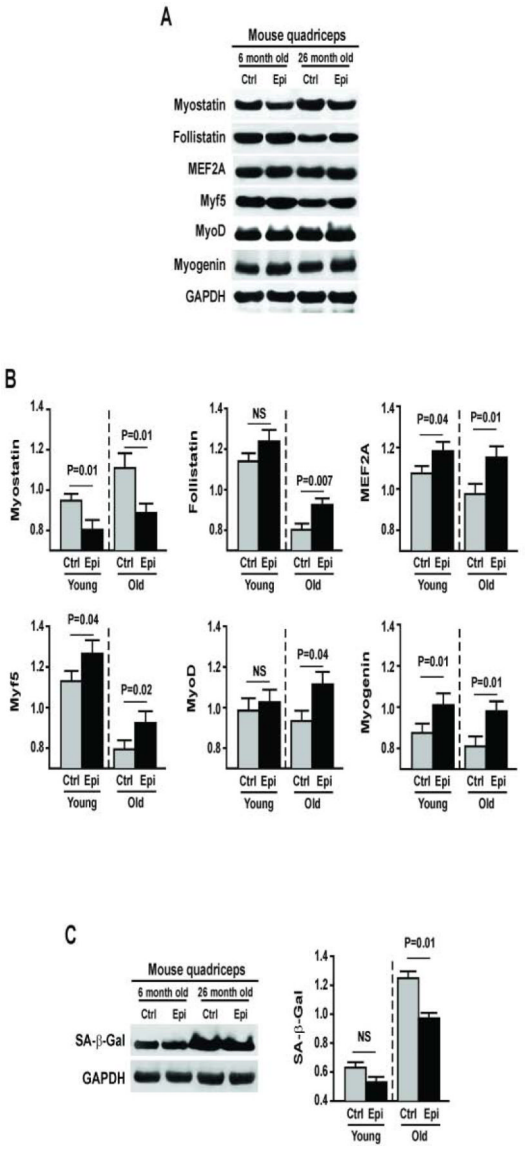

Figure 1.

Differences observed in SkM growth/differentiation and SA-β-Gal protein levels in young and old control or (−)-epicatechin treated mice. Panel **A** shows Western blot images observed in myostatin, follistatin, MEF2A, Myf5, MyoD, myogenin. Panel **B** plots the mean ± SEM densitometric units of the protein of interest normalized over GAPDH values. Panel **C** denotes differences observed in senescence associated-β-Gal protein levels. (n=5/group).

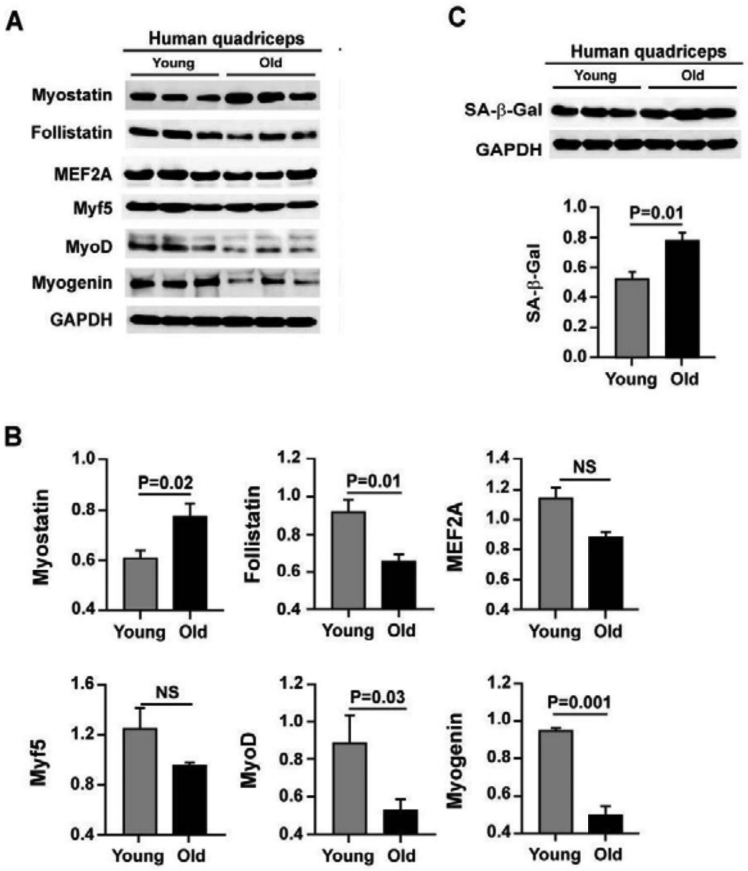

Figure 2.

Differences observed in markers of muscle growth, in older vs. young human subjects, and effects of 1 week (−)-epicatechin treatment on strength and SkM growth markers in middle age subjects. Panel **A** shows Western blot images for myostatin, follistatin, MEF2A, Myf5, MyoD, myogenin protein levels in human quadriceps from young vs. old subjects. Panel **B** plots the mean ± SEM densitometric units of the protein of interest normalized over GAPDH values. Panel **C** denotes differences observed in senescence associated-β-Gal protein levels. (n=5/group).