International Journal of Stem Cells Vol. 3, No. 1, 2010

ORIGINAL ARTICLE

The Effect of Nutritional Supplements on Muscle-Derived Stem Cells *in vitro*

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Postnatal muscle stem cells, recognized as myogenic satellite cells, were isolated from sheep skeletal muscle and used in these experiments. Forty-one different metabolic compounds that are commonly found in commercially-available oral supplements were exposed to primary muscle stem cell cultures, in an effort to ascertain whether any one compound could alter satellite cell proliferation or differentiation (a first step towards elucidating the metabolomics or nutrigenomics of these stem cells). These compounds included energetic moieties, amino acid analogs, fatty acids and analogs including different forms of conjugated linoleic acid, minerals and mineral conjugates, insect hormones, caffeine, plant extracts, and extracts from over-the-counter supplements, and were obtained by key manufacturers in a form that would be commercially available. The compounds were sterilized and then exposed to myogenic satellite cell cultures at different levels (ranging from toxic to physiologic) to ascertain if there would be an effect. The results suggested that exposure of satellite cells to only a few compounds resulted in any measurable effect(s). Ten compounds elicited increases in proliferation, and four compounds promoted increases in differentiation. These results suggest avenues for the exploration of enhancing muscle stem cell activity of interest for muscle wasting disorders, sarcopenia of aging and physical performance.

Keywords: Muscle stem cells, Satellite cells, Dietary supplements, Performance, Assay

Introduction

Myogenic satellite cells (SC) are postnatal skeletal muscle stem cells (1, 2). Without the activity of myogenic SC, postnatal skeletal muscle hyperplasia, hypertrophy, or repair would be impossible (3). Satellite cell fusion (4-6) with adjacent myofibers provides the nuclei required for regulating myofibrillar protein accretion (7), and the cellularity of these cells is required for replacing damaged myofibers (1). The history of the development of the ovine satellite cell system, *in vitro*, (1, 4-10), and the rationale for its use in the types of studies described, herein, have been reported (11-18).

Understanding the linkage between SC activity and dietary components is a timely area of research. Whether simply elucidating the influence of dietary constituents on satellite cell proliferation and differentiation, or generating complex knowledge regarding the influence of diet on gene regulation (nutrigenomics; 19) or metabolism (metabolomics; 19), information gained in this arena will help us understand the complex regulation of stem cells found in skeletal muscle. As a consequence, the present study was undertaken to gain additional knowledge about the mechanisms through which individual dietary supplements regulate SC activity *in vitro*.

Accepted for publication March 21, 2010
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Materials and Methods

Materials

The compounds were obtained from companies that

Table 1. A list of the tested compounds and the corresponding manufacturers

Compound	Manufacturer
L-arginine base	P.L. Thomas & Co
Disodium 5′-inosinate	P.L. Thomas & Co
Disodium 5′-guanylate	P.L. Thomas & Co
Disodium guanosine 5′-monophosphate	P.L. Thomas & Co
Disodium inosine 5′-monophosphate	P.L. Thomas & Co
L-carnosine	P.L. Thomas & Co
L-citrulline	P.L. Thomas & Co, Amax NutraSources, Inc.
ATP	Kyowa Hakko Kogyo Co.
L-ornithine aspartate	Kyowa Hakko Kogyo Co.
Citicoline	Kyowa Hakko Kogyo Co.
Caffeine anhydrous powder	Stauber Performance Ingredients
L-glutamine powder	Stauber Performance Ingredients
L-arginine HCl	Integrity Nutraceuticals International
Pinitol	Integrity Nutraceuticals International
β-hydroxy-β-methyl butyrate calcium salt monohydrate	Integrity Nutraceuticals International
Evodiamine	Integrity Nutraceuticals International
Ecdysterone	AIDP, Inc.
N-acetyl-L-cysteine	AIDP, Inc.
Taurine	AIDP, Inc.
SoLite™ Blend	Designed Nutritional Products
Disodium inosinate I003-XX-SOD-PD-RRR	Triarco Industries
Chondroitin sulfuric acid	Novel Ingredient Services
Vita Blue Wild Blueberry Extract	Future Ceuticals
Humanofort 17-keto sulfate	Fitness Enterprise
Acetyl carnitine hydrochloride	Biosint USA
Carnisheild™ B94	Biosint USA
CDP-choline monosodium salt	Pro. Bio. Sint.
UMP disodium salt	Pro. Bio. Sint
Trimethyl Glycine TMG 20	no manufacturer
Diachrome	Nutrition 21
Sulbutiamine T-207	TSI
Ubidecarenone 5%	Nisshin Flour Milling
Calcium-ketoglutarate	EAS "CGK"
Magnesium Pidolate	Kelatron
Aspartate arginine	Sarcenor-Lab. Sarget Pharma
CLA Ultra Lean	Weider Body Shaper
Clarinol G-80	Loders Croklaan
Marinol D50 Omega 3	Loders Croklaan
Clarinol Gelcaps	Loders Croklaan
NKO™ 100% Krill Oil	Neptune Pharmaceuticals, Inc.
BioAstin Oleoresin 5% Astaxanthin	Cyanotech Corporation

supply manufacturers of dietary supplement products (Table 1). Dulbecco's Modified Eagle Medium (DMEM), horse serum (HS), penicillin/streptomycin, and gentamicin were purchased from Invitrogen/Life Technologies. Pigskin gelatin (porcine skin, type A), Giemsa stain (Accustain), trypsin, KCl, KH_2PO_4, NaCl, Na_2HPO_4, and $Na_2EDTA \cdot 2H_2O$ were purchased from Sigma Chemical Company. The cell culture plates and flasks were purchased from Nalge Nunc™.

Cells

The satellite cells used for the present studies were exactly as described previously (13, 14). The basal medium for proliferation consisted of DMEM+2% HS whereas the differentiation medium consisted of DMEM+0.5% HS. The treatment compounds were added to the basal medium at four separate levels. The treatment medium was changed every 24 hr until a pre-determined endpoint was reached (96 h for proliferation, 120 h for differentiation). At this time, the cultures were removed from incubation, fixed with methanol, stained with Giemsa, and then counted to determine the effects of treatment on cell proliferation and/or differentiation (13, 14).

Test compounds

Water soluble compounds were added to plain DMEM to make a 0.1% stock solution. Six compounds (Evodiamine, Diachrome, Carnisheild™ B94, SoLite™ Blend, Humanofort 17-Keto Sulfate™, and Vita Blue Wild Blueberry Extract) had high particulate matter after suspending in DMEM and were centrifuged for 5 min (SoLite™ Blend, Humanofort 17-Keto Sulfate™, Carnisheild™ B94, and Vita Blue Wild Blueberry Extract) or 10 min (Evodiamine, Diachrome) at 2700 rpm (1500×G using a horizontal rotor; Beckman TJ-6 centrifuge) and the supernatant retained. The pH of all the stock solutions was returned to 7.08 and sterile-filtered first through a 0.45 μm pore syringe filter to remove larger particulate matter and then sterile-filtered through a 0.2 μm pore syringe filter. For all compounds, the first treatment level was a 1 : 10 dilution of the stock solution and the remainder of the treatments consisted of three 1 : 100 serial dilutions. The control for each compound consisted of basal media without the test compound.

Fatty acids and oils were either added to DMSO or to 100% ethanol to aid in solubility. Marinol D50 Omega 3, Clarinol G-50, and NKO™ 100% Krill Oil were suspended in DMSO (2.5 ml compound in 5 ml DMSO) to make a saturated solution. Clarinol G-50 and NKO™ were removed from the capsule before adding to the DMSO.

The suspensions were filtered through a 0.45 μm pore syringe filter and then sterile-filtered through a 0.2 μm pore syringe filter rated for DMSO to make a stock solution. The first treatment level was a 1 : 10 dilution of the stock medium. The remainder of the treatments consisted of three serial 1 : 100 dilutions. It was determined that the highest treatment level contained 0.067 μl of DMSO/ml medium; therefore this amount of DMSO was added to the controls. The controls consisted of basal media+0.67 μl DMSO/ml basal medium. Clarinol G-80 was added to 100% ethanol to yield a 10% stock solution and sterile-filtered through a 0.2 μm pore syringe filter. The solution was returned to pH 7.08. The first treatment was a 1 : 100 dilution of the stock (for a 0.1% solution) in basal medium, and the remaining treatments were three serial 1 : 100 dilutions. The contents of one CLA Ultra Lean capsule were removed with a needle and syringe, added to 7.8 ml of 100% ethanol, and filtered through a 0.2 μm pore syringe filter to make a 5% stock solution. The first treatment is a 1 : 50 dilution of this stock into basal medium (for a 0.1% solution), and the remaining treatments were three 1 : 100 serial dilutions. BioAstin Oleoresin 5% Astaxanthin/95% safflower oil was added to 100% ethanol for a stock solution and sterile filtered through a 0.2 μm pore syringe filter. The first treatment level was a 1 : 1,000 dilution of the stock, and the remaining three treatments were serial 1 : 100 dilutions. The controls for these compounds consisted of basal medium with 1 μl of 100% ethanol per milliliter of medium added. The concentrations of all the compounds ranged from below physiologically relevant to toxic levels.

Serum-free proliferation

The compounds that were determined to have a statistically significant treatment effect in serum-containing medium were selected for further testing in a basal, serum-free defined medium: ovine defined medium (ODM) for proliferation (REF) and ITT-C (insulin, transferrin, triiodothyronine, cortisol) for differentiation (18). Creatine monohydrate (CM) has been shown to affect satellite cell differentiation (18); therefore CM was added to the defined media to test for an additive effect.

Statistical analysis

All screening experiments were performed and evaluated exactly as previously described in our prior study on ergogenic compounds (18). The experiments consisted of four wells per treatment point and were performed twice for a total of eight wells per treatment. At the termination of the experiments, all the cultures were counted, data were collated and reduced, treatment differences were detected with a one-way ANOVA and post-tests were conducted using Tukey's pairwise comparisons. The statistical analysis was performed using Graphpad Instat version 3.05. The data was presented as Mean±SE and considered significant when ($p<0.05$).

Results

Proliferation and Differentiation

The morphology of all the cells was normal. The highest levels of fatty acid compounds caused cell death. The results of the proliferation assays are summarized in Table 2. Significant increases in cell numbers compared to control values ($p<0.05$) were found in 10 compounds: Disodium 5'-inosinate, Disodium guanosine 5'-monophosphate, Disodium inosine 5'-monophosphate, L-citrulline, ATP, Caffeine anhydrous powder, Ecdysterone, Humanofort 17-keto sulfate, CLA Ultra Lean, Clarinol G-80. For differentiation, the morphology of all the cells was normal. The highest levels of fatty acid compounds caused cell death. Significant increases in SC differentiation compared to control values ($p<0.05$) were found for four compounds: disodium 5'-guanylate, disodium inosine 5'-monophosphate, taurine, and disodium inosinate I003-XX-SOD-PD-RRR.

Discussion

In the present study, we attempted to gain knowledge about the regulation of postnatal skeletal muscle stem cells (3, 7) as influenced by a variety of different nutraceutical agents (18) using an assay system previously validated (11-18). Satellite cells are important for normal skeletal muscle growth (increase in muscle mass) and repair (3, 7). Interest in stimulating muscle mass encompasses three major areas: 1) muscle wasting disorders; 2) sarcopenia of aging and 3) athletic or physical performance.

In addition to consuming adequate caloric and protein intake during resistance training (20), creatine supplementation is generally regarded as effective for increasing muscle mass and strength (20, 21). Previous work with muscle SCs has found that creatine had consistent effects on satellite cell differentiation *in vitro* (7), which may partly explain the ability of exogenous creatine to enhance muscle mass and strength. Other compounds, mostly nucleic acid salts and taurine, have been shown to affect muscle satellite cell proliferation and/or differentiation in this study (Table 2), adding to a list of nutrients found in dietary supplements that have the potential to positively affect muscle mass (2, 5-8). Other nutrients with

Table 2. The effects of compounds tested on satellite cell proliferation and differentiation

Compound	Proliferation	Differentiation[a]
L-arginine base	ns[b]	ns
Disodium 5′-inosinate	10^{-4}, 10^{-6}	ns
Disodium 5′-guanylate	ns	10^{-4}, 10^{-8}
Disodium guanosine 5′-monophosphate	10^{-6}	ns
Disodium inosine 5′-monophosphate	10^{-4}, 10^{-6}	10^{-8}
L-carnosine	ns	ns
L-citrulline	10^{-4}	ns
ATP	10^{-6}	ns
L-ornithine L-aspartate	ns	ns
Citicoline	ns	ns
Caffeine anhydrous powder	10^{-4}	ns
L-glutamine powder	ns	ns
L-arginine HCl	ns	ns
Pinitol	ns	ns
β-hydroxy-β-methyl butyrate calcium salt monohydrate	ns	ns
Evodiamine	ns	ns
Ecdysterone	0.1%	ns
N-acetyl-l-cysteine	ns	ns
Taurine	ns	10^{-6}, 10^{-8}
Solite^{tm} blend	ns	ns
Disodium inosinate I003-XX-SOD-PD-RRR	ns	10^{-8}
Chondroitin sulfuric acid	ns	ns
Vita blue wild blueberry extract	ns	ns
Humanofort 17-keto sulfate	10^{-4}	ns
Acetyl carnitine hydrochloride	ns	ns
Carnisheild^{tm} B94	ns	ns
CDP-choline monosodium salt	ns	ns
UMP disodium salt	ns	ns
Trimethyl glycine TMG 20	ns	ns
Diachrome	ns	ns
Sulbutiamine t-207	ns	ns
Ubidecarenone 5%	ns	ns
Calcium-ketoglutarate	ns	ns
Magnesium pidolate	ns	ns
Aspartate arginine	ns	ns
Bioastin oleoresin 5% astaxanthin	ns	ns
CLA Ultra Lean	0.0000001	ns
Clarinol G-80	0.001, 0.0000001	ns
Marinol D50 Omega 3	ns	ns
Clarinol Gelcaps	ns	ns
NKO^{TM} 100% Krill Oil	ns	ns

[a]Differentiation measured as the presence of three or more nuclei within one cell membrane, [b]ns=no significance between treatment means at $\alpha=0.05$.

effects on satellite cells included certain fatty acids, such as conjugated linoleic acid (CLA). Fatty acids (FA) and FA derivatives are thought to exert effects through gene regulation (22), perhaps mediated via the nuclear protein peroxisome proliferator activated receptor (PPAR) to mediate the differentiation and subsequent filling of adipocytes (23). Similarly, for muscle cells linoleic acid has been determined to enhance the formation of myotubes from isolated myogenic satellite cells (24) and in L6 myoblast cultures (25). Additional experimentation with conjugated linoleic acids (CLA) and L6 myoblasts showed both a proliferative and/or a differentiative effect depending on the dose of CLA and the isomer of CLA to which the cells were exposed (25), whereas protein complexes like Humanofort (a mixture of growth factors and other proteins; (26) had only minor effects on SC proliferation.

What is the ramification of individual dietary components altering the activity of satellite cells *in vitro*? The effects *in vitro* may/may not be operative *in vivo* after oral administration. Digestion, absorption, and metabolism all influence the concentrations and identities of ingested nutrients presented to satellite cells. The relevance of sheep muscle to human muscle is also another variable to consider, but a variety of different animals have been used for satellite cell research with a final reference towards providing knowledge parallel to human physiology (3). Nevertheless, despite limitations, the research presented here is a starting point for the evaluation of dietary supplement ingredients as legitimate agents for enhancing muscle activity.

The effect on muscle SCs is one possible mechanism for encouraging muscular hypertrophy. In theory, affecting SCs should also have activity in non-athletic muscle activity, such as muscular wasting or sarcopenia of aging. In general, the results from muscle SCs on proliferation and differentiation are compatible with other data for compounds with the potential utility to support muscle repair, hypertrophy and hyperplasia. As the world population increases, so will infirmity and decreased functional ability of the elderly and infirmed. Knowledge of which compounds might aid treating muscle wasting, or which compounds may enhance future stem cell therapies will be increasingly important. The logical place to begin looking for treatments for maintaining mobility and load-bearing capability is the stem cells (satellite cells) of skeletal muscle. Collectively, these data provide basic knowledge about stem cells in skeletal muscle and suggest that satellite cells may be influenced by metabolites and other (potential) nutraceutical agents.

Currently, only the athletic performance area, including the body-building arena, makes use of dietary supplements targeted towards building muscle. In recent years, this field of use has seen some products sold as dietary

supplements that contain ingredients that are in contradiction of the Dietary Supplement Health and Education Act (which governs dietary supplements in the United States). A need for ingredients in dietary supplements that may, in theory, aid muscle mass accretion without the use of banned substances (such as anabolic steroids) exists and was an impetus for the research described herein. However, the resulting data are not limited to this application but has wider reaching implications.

Potential Conflict of Interest

The authors have no conflicting financial interest.

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