7-12-2014

Effects of dietary stimulators of metabolism and mitochondrial biogenesis in vitro and in vivo: Implications for metabolic disease

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Effects of dietary stimulators of metabolism and mitochondrial biogenesis *in vitro* and *in vivo*: Implications for metabolic disease

by

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Bachelor of Science: Nutrition and Dietetics, University of New Mexico

Master of Science: Nutrition, University of New Mexico

DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy: Physical Education, Sports and Exercise Science

The University of New Mexico

Albuquerque, New Mexico

May 2014

Dedication

I dedicate this work in its entirety to the wonderful people who have tirelessly supported me and thoughtfully contributed to the completion of this research. I would like to thank my co-authors and laboratory partners Randi Garcia-Smith and Nicholas Gannon for their ongoing efforts to make research fun and exciting, as well as accurate and informative. I would also like to thank the mentors who welcomed me into their laboratories and lives; my success is a product of their expertise and devotion to students. Dr. Kravitz, thank you for always providing inspiration and support with your seemingly infinite kindness and positive attitude. I would also like to thank Dr. Mermier, who has not only mentored this work, but also provided numerous opportunities for me to excel in exercise physiology research. I will always cherish the time I spent in the ex phys lab, for both the academics and friendships I have developed. I had a great time! I would also like to thank Dr. Bisoffi for his kindness in welcoming me into his lab, which has allowed me to pursue a life goal of becoming a molecular researcher. You are a remarkable scientist, and I hope it comes as no surprise, but I aspire to be like you. Thank you! I would also like to thank Dr. Trujillo, whom has also welcomed me into her lab. Your mentorship has provided me with professional development and insights that will last throughout my career. I also have to thank you for reminding me that there is more to life than science. I think that you have achieved the perfect balance of work and recreation, a balance of which I hope to experience in my own life. Lastly, I would like to thank Dr. Conn. You have served as my mentor, supervisor, teacher, and life coach for nearly a decade. No other person has influenced my professional development more than you. I can't begin to thank you for all of your ongoing support and advice during our work together. I am truly thankful for having the opportunity to work with you. It has been one of my life's greatest honors and privileges. Thank you!

I would also like to thank several family members and friends. I would like to thank my fabulous sisters Amanda and Roxanne as well as my brother Rob, his wife Amy and their three wonderful children. To my parents, I thank you for all of your ongoing support. Dad, your words and wisdom bring me peace. Mom, although we are unable to discuss the intricacies of my life daily as we used to, I am sure that you know how essential you were to my development and success. I love and miss you. You were the best!

Most of all, I would like to thank my wonderful wife Loren. Sweetie, you're the best part of my life. I admire the way that you always put the needs of others ahead of your own needs. Without you, none of my success would have been possible. You are my biggest support, and the best person I know. Thank you for your support and for always making me want to be a better person. I love you!

Thank you ALL,

Working with you has been one of my life's greatest pleasures

Acknowledgments

We would like to thank the University of New Mexico Department of Biochemistry and Molecular Biology and the Department of Health, Exercise and Sports Science for their assistance in this work. We would also like to thank the human test subject volunteers for their time and willingness to participate in our study. Additionally, we would also like to thank the shared facilities available through the University of New Mexico Health Sciences Center: Flow cytometry data was generated in the Flow Cytometry Shared Resource Center supported by the University of New Mexico Health Sciences Center and the University of New Mexico Cancer Center. Images were generated in the University of New Mexico & Cancer Center Fluorescence Microscopy Shared Resource, funded as detailed on: http://hsc.unm.edu/crtc/microscopy/acknowledgement.shtml. All authors and contributors declare no conflict of interests.

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Abstract

Background: Commercially available dietary products advertised to promote weight loss are an under researched but heavily purchased commodity in the United States. Despite only limited evidence, interest in dietary supplements continues to increase. Obesity is an increasingly prevalent and preventable morbidity with multiple behavioral, surgical and pharmacological interventions currently available. Commercially available dietary supplements are often advertised to stimulate metabolism and cause rapid weight and/or fat loss, although few well controlled studies have demonstrated such effects. This work uniquely summarizes the current evidence evaluating the efficacy of several over-the-counter thermogenic products for their effects on resting energy expenditure. Additionally, this work outlines the important therapeutic

benefits provided by dietary stimulators of metabolism and peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1) in skeletal muscle. Our work uniquely describes the effects of a commercially available dietary supplement on resting metabolic rate in humans as well as the metabolic and biochemical effects *in vitro*. **Methods**: Human rhabdomyosarcoma cells (RD) and mouse myoblasts (C2C12) were cultured under standard conditions and treated with various doses of a commercially available supplement (RF) for various durations and assessed for changes in metabolism, metabolic gene expression and mitochondrial content. Additionally, human subjects ingested either placebo or RF in a double-blind placebo controlled fashion and metabolic rate and blood pressure were measured at 3 time points for 3 hours post-ingestion. **Results**: RF enhanced metabolism, metabolic gene expression, and mitochondrial content in both cell models. RF also enhanced energy expenditure in human male subjects without altering substrate utilization. RF also significantly increased systolic blood pressure. **Conclusion**: RF appears to increase metabolism immediately following ingestion, although additional research is needed to assess safety and efficacy for human weight loss.

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Chapter 1

Effects of Commercially Available Dietary Supplements on Resting Energy Expenditure: A Brief Report

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The final publication of this article is available at:

http://www.hindawi.com/isrn/nutrition/2014/650264/

Abstract

Commercially available dietary products advertised to promote weight loss are an under researched but heavily purchased commodity in the United States. Despite only limited evidence, interest in dietary supplements continues to increase. This work uniquely summarizes the current evidence evaluating the efficacy of several over-the-counter thermogenic products for their effects on resting energy expenditure. Currently, there is some evidence suggesting dietary products containing select ingredients can increase energy expenditure in healthy young people immediately following consumption (within 6 hours). It is unclear if supplement-induced increases in metabolic rate provide additional benefit beyond that provided by dietary constituents that contain similar ingredients. It is also unclear if dietary supplements are effective for weight loss in humans.

Key Words: Thermogenic Supplements, Fat Burners, Metabolic Rate, Green Tea, Caffeine, Capsaicin

Introduction

Obesity has rapidly become a leading cause of death without a foreseeable resolution in the near future. Most clinicians believe the combination of food overconsumption with sedentary lifestyle synergistically promote weight gain and obesity. The importance of restrictive dietary practices in combination with physical activity are of undeniable importance for weight loss and general health [1, 2], however, the role of genomics and corresponding interactions with dietary and exercise practice still remain largely ill-defined as the field is still in its infancy [3]. A variety of therapies are currently available to combat obesity, however recent trends in obesity prevalence provide strong evidence that current interventions are insufficient to effectively slow the development of obesity and related comorbidities [1-3].

Of increasing interest is the role which food chemicals and dietary components may play in obesity therapeutics. In obesity research, several classes of chemicals including methylxanthines, polyphenols, capsaicin (capsaicinoids/capsinoids), polyunsaturated fats, and many other components found in food have shown some promise in promoting a metabolic advantage for weight loss [4, 5]. As a result of preliminary data supporting some of the aforementioned ingredients, producers of commercially available dietary supplements often include one or many of these components in their products [5]. Dietary supplements are sold for a variety of purposes, including weight loss, and many ingredients are promoted to have specific benefits such as increased thermogenesis.

Dietary supplements are unique because unlike pharmaceutical agents, they do not require close regulation of content, function, or safety prior to consumption by humans [6] and are generally not recommended by healthcare professionals [7]. Because of limited regulation, it is not uncommon for single ingredients to be promoted for a promiscuous variety of unsubstantiated functions and health benefits. Although subjected to limited regulation, it is mandated that all

dietary supplements be marked with a disclaimer stating the lack of support by the FDA for all claims. Supplements sold as thermogenic products for weight loss and/or energy augmenters are often promoted to cause rapid weight loss, often times independent of calorie restriction or physical activity. Despite resistance from healthcare professionals, consumer interest in dietary supplements continues to surge [8]. According to NHANES questionnaire data from roughly 12,000 participants, approximately 50% of those surveyed used dietary supplements in the past 30 days [8]. Use was most common among 20-30 year old participants, and more common in women than in men [8]. Interestingly when asked to select the motivating factor for supplement consumption, "weight loss" or "get more energy" were reported as the motivation for approximately 14% of participants, both of which were also more common in women participants [8]. This work seeks to summarize current research evaluating commercially available dietary supplements sold as stimulators of thermogenesis and increased metabolic rate leading to weight loss.

Individual Ingredients as Metabolic Stimulators

Caffeine and xanthine metabolites

Of the available dietary supplements marketed for weight loss, many contain a blend of ingredients that includes caffeine. Caffeine has previously been shown to dose-dependently heighten resting energy expenditure in adult humans, both normal and overweight [5, 9-14]. Despite some conflicting data, it is generally accepted that caffeine effectively stimulates the central nervous system and increases metabolic rate in humans [14-16]. Caffeine functions through inhibition of phosphodiesterase (PDE) and through stimulation of adenosine receptors, leading to accumulation of intracellular 3,5-cyclic-adenosine monophosphate (cAMP) which is metabolically excitatory for cells [11, 12, 14].

β_2–adrenergic receptor agonists

Like caffeine, stimulators of β_2–adrenergic receptors such as ephredra/ephedrine have been another primary component found in supplemental thermogenic products [9, 11, 14]. Following the restriction of ephedra use, similar chemical analogs such as synephrine from bitter orange extract became widely accepted replacements [17-20]. β_2–adrenergic receptor agonists work to increase cAMP biosynthesis while caffeine inhibits the breakdown of cAMP by PDE [14]. Additionally, salicylates inhibit prostaglandins further allowing the accumulation of cAMP [14]. Taken together, caffeine, β_2–adrenergic receptor agonists, and salicylates function to synergistically increase cAMP accumulation and metabolic rate [14].

Green tea extract polyphenols

Several other dietary stimulators of metabolism are thought to function by augmenting the accumulation of cAMP [14]. Green tea extract is rich in polyphenols and is heavily promoted for health benefits including increased metabolism [4, 12-14, 21-28]. Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC), are believed to cause most of green tea's beneficial effects [5]. In other teas such as black tea, thearubigins and theaflavins share similar effects to those found in green tea [4, 29]. Green tea extract appears to offer some thermogenic effect when coupled with caffeine resulting in increased energy expenditure [14, 22, 30]. In contrast, isolated green tea polyphenol ingestion independent of caffeine does not appear to increase metabolic rate, however does increase indicators of fat metabolism in some populations [14, 22, 30].

Green tea is purported to function by inhibiting the degradation of β_2–adrenergic receptor agonists such as norepinpherine by the enzyme catechol O-methyltransferase (COMT), thereby increasing intracellular cAMP [4, 12]. Again, the combination of green tea polyphenols with caffeine cause a synergistic effect further increasing cAMP. Recently, the effects and

mechanisms of green tea extract in human metabolism were reviewed and it was suggested that the mechanism of COMT inhibition is possibly a product of *in vitro* experimental conditions, because COMT inhibition experiments have yet to identify a specific catechin inhibitor, or determine if the active polyphenol is an inhibitor, a substrate of COMT, or a combination [31]. Additionally, the mechanisms of the hypothesized downstream effect of COMT inhibition resulting in heightened fat metabolism has yet to be elucidated experimentally [31]. In addition to COMT inhibition, it is theorized that effects of green tea extract are also a function of metabolic gene activation. Specifically, green tea may function to inhibit adipogenic genes such as peroxisome proliferator-activated receptor (PPARγ), and sterol regulatory element binding protein-1c (SREBP-1c), and increase the expression of genes that increase energy expenditure and mitochondrial biogenesis including nuclear respiratory factors (NRF), mitochondrial uncoupling protein-3 (UCP3), peroxisome proliferator-activated receptor (PPARα), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), leading to increased oxidative and metabolic capacities [31].

Capsaicinoids and capsinoids

Another common constituent of thermogenic products are the capsaicinoids/capsinoids, components found in spicy foods such as chili pepper and cayenne [32, 33]. Available data suggests that supplemental capsaicinoids/capsinoids effectively increase resting energy expenditure, although findings have been inconsistent [32-34]. Capsaicinoids are believed to function by increasing catecholamine release, heightening sensitivity to circulating catecholamines, or a combination thereof [33]. Figure 1 summarizes the proposed mechanisms of the abovementioned individual ingredients commonly found in commercially available thermogenic products.

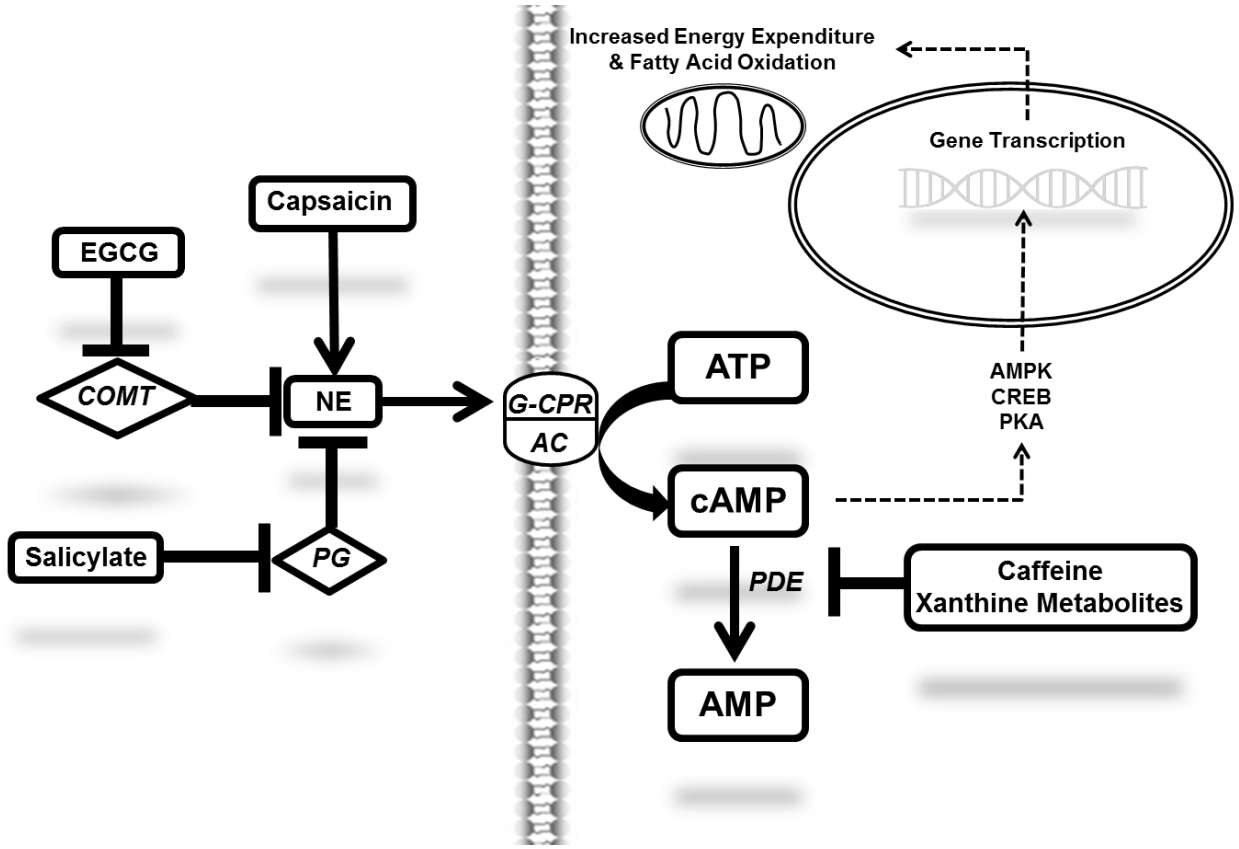

Figure 1 Summary of proposed mechanisms of common ingredients in thermogenic products.

Abbreviations: Adenylate Cyclase (AC), Adenosine Monophosphate (AMP), 3,5-cyclic-Adenosine Monophosphate (cAMP), 5'Adenosine Monophosphate-Activated Protein Kinase (AMPK), Adenosine Triphosphate (ATP), Catechol O-methyltransferase (COMT), cAMP-Related Element Binding Protein (CREB), Epigallocatechin Gallate (EGCG), G-Coupled Protein Recepetor (G-CPR), Prostaglandin (PG), Protein Kinase A (PKA), Nor-epinepherine (NE), Phosphodiesterase (PDE).

Propriety and Commercially Available Thermogenic Products

Proprietary products

Several previous investigations have identified that commercially available products increase resting energy expenditure in a variety of subjects, most frequently using young healthy subjects (Table 1). Most commonly these products contain several of the following: caffeine, green tea, capsaicin, and catecholamine-like compounds (synephrine). In general, most investigations evaluated changes in energy expenditure within 6 hours of consumption. While the magnitude of the individual supplement's effect on energy expenditure seems to vary depending on the

ingredients consumed, the increase in energy expenditure is similar to previous observations using individual ingredients such as caffeine, ranging from 50 to 200 kcals per hour immediately following ingestion. Although this increase is experimentally significant, it must be stressed that the effect is time-dependent and energy expenditure appears to return to basal levels 6 hours following consumption. Previous acute (24 hours or less) investigations with caffeine alone or green tea with caffeine showed approximately a 4% increase in 24 hour metabolic rate [5, 10, 12, 14] which approximates the increases seen with selected thermogenic products. Extended treatment for up to 8 weeks has also shown increased resting energy expenditure, suggesting that regular green tea consumption with select stimulants can continuously cause an increase in metabolism [35, 36].

Table 1 Investigations evaluating the effects of thermogenic products on resting energy expenditure (REE) following acute (24 hours or less) and extended (longer than 24 hours)

Effects of acute consumption of dietary supplements on energy expenditure

Subjects	Ingredients	Findings	Author
Obese men (N = 19)	Green tea extract 750mg L-tyrosine 609mg Caffeine 151mg Cayennea 225mg Calcium 1965mg	Supplement containing bioactive food ingredients increased daily REE by 200 kJ or 2% (48 kcal)	Belza 2005 [34]
Healthy men and women (N = 20)	Caffeine Anhydrous Toothed Clubmoss (Huperzia Serrata) Yerba Mate (Llex Paraguariensis) 3'-5'-CAMP (3'-5'-Cyclic Adenosine Monophosphate) Synephrine HCL R-Beta-Methylphenylethylamine N-Mehtyl-B-Phenylethylamine Yohimbe Methyl-Hordenine	Increased REE 59 ± 26 kcal·6 hrs, serum epinephrine, norepinepherine, glycerol, systolic and diastolic BP	Bloomer 2009 [37]
Healthy men and women (N = 10)	Caffeine Anhydrous Toothed Clubmoss (Huperzia Serrata) Yerba Mate (Llex Paraguariensis) 3'-5'-CAMP (3'-5'-Cyclic Adenosine Monophosphate) Synephrine HCL R-Beta-Methylphenylethylamine N-Mehtyl-B-Phenylethylamine Yohimbe Methyl-Hordenine	Increased REE, increased HR no change in BP	Hoffman 2009 [38]
Healthy men and women (N = 12)	Caffeine Anhydrous Toothed Clubmoss (Huperzia Serrata) Yerba Mate (Llex Paraguariensis) 3'-5'-CAMP (3'-5'-Cyclic Adenosine Monophosphate) Synephrine HCL R-Beta-Methylphenylethylamine N-Mehtyl-B-Phenylethylamine Yohimbe Methyl-Hordenine	Increased REE 45, 60, and 120 minutes post ingestion with no change in HR or BP	Jitomir 2008 [39]
Healthy men and women (N = 8)	Caffeine anhydrous, guarana, yerba mate green tea extract, L-carnitine L-tartrate, pathothenic acid, chromium picolinate, and proprietary blends containins , AssuriTea™ Green Tea Extract, Salvia sclarea, raspberry ketones and Capsicum Annum extract, plus l-tyrosine, salix alba, zingiber officinale, focus vesiculosus, panax ginseng, and BioperineW	Increased REE 60, 120, 180, 240 minutes post ingestion	Outlaw 2013 [40]
Healthy men and women (N = 28)	Supplement Caffeine 200 mg, Capsicum extract* 33.34 mg, Niacin 20 mg, Bioperine 5 mg	Increased REE 50 minutes post consumption with elevated HR and BP	Ryan 2009 [32]
40 male and 40 female healthy young subjects	1 mg capsinoids in 199 mg of rapeseed oil and medium-chain triglycerides	No change in REE or weight but increased fat oxidiation	Snitker 2009 [41]
10 healthy subjects per treatment	600 mg naringin, 50 mg *p*-synephrine	Increased REE 129 kcal	Stohs 2011 [17]
	100 mg hesperidin, 50 mg *p*-synephrine, 600 mg naringin	Increased REE 183 kcal	
	1000 mg hesperidin, 50 mg *p*-synephrine, 600 mg naringin	Increased REE 79 kcal	
8 male and 10 female healthy young subjects	442 mg of a proprietary: 100 mg of caffeine, 230 mg of green tea extract, L-tyrosine, L-taurine, Chocamine, white willow extract, yohimbine-HCl, and vinpocetine	Increased REE 60, 120, 180 minutes post ingestion	Wilborn 2009 [42]
Healthy men and women (N = 60)	caffeine, citrus aurantium, garcinia, cambogia and chromium polynicotinate	significantly increased REE at 60, 120 and 180 min	Dalbo 2008 [43]
18 healthy young men	1.5 mg capsinoids in 199 mg of rapeseed oil and medium-chain triglycerides	EE increased by 15.2 kJ/h (BAT+) (5 kcals/hour) groups 1 hours post ingestion	Yoneshiro 2012 [44]

Effects of extended consumption of dietary supplements on energy expenditure

Subjects	Ingredients	Findings	Author
Healthy men and women (N = 8)	pantothenic acid, 40 mg; green tea leaf extract 200 mg; guarana extract (198 mg of caffeine), 550 mg; bitter orange (9 mg of synephrine), 150 mg; white willow bark extract (7.5 mg of salicin), 50 mg; ginger root, 10 mg; and proprietary blend (L-tyrosine, Lcarnitine, and naringin), 375 mg, phenylephrine (20 mg)	Increased REE with increased body weight	Greenway 2006 [36]
Obese subjects (N = 80)	Green tea extract 750mg L-tyrosine 609mg Caffeine 151mg Cayennea 225mg Calcium 1965mg	Supplement increased REE by 87.3 kJ (21 kcal) sustained for 8 weeks with reduced body fat mass	Belza 2007 [35]

Despite evidence that several of the single ingredients previously described have been shown to increase metabolic rate, there is some question about the efficacy of advertised dietary products for weight reduction. Hasani-Ranjbar and colleagues selectively identified dozens of studies which implicate natural products in the reduction of body weight in humans [6]. The investigations reviewed ingredients frequently found in dietary supplements including caffeine, ephedra, and a variety of herbal agents, many of which function in part as antioxidants [6]. These studies included experimental designs lasting from as little as 4 weeks to up to 9 months without controlling for exercise and/or concurrent energy restriction. In general, there appears to be some evidence that thermogenic products may partially aid in weight loss, although it is difficult to identify the magnitude because of between-study variables.

Limitations to Current Observations

Individual ingredients

One possible contributor to discrepancies within the literature regarding efficacy of individual ingredients is experimental variability. Multiple confounds exist in energy expenditure measurements such as duration of measurement, type of analytical and measurement software, duration of measurement post ingestion of stimulant, and the amount of purported metabolic stimulator consumed. An additional issue is that much of the original measurements were conducted several decades ago. Moreover, significant biological differences between demographics may also contribute to varied response across similar studies.

Commercial products

Proprietary blends found within dietary supplements have numerous issues that are not encountered when testing pharmaceutical grade single chemicals (such as caffeine). For example, dietary supplements may vary in content from one lot to the next, suggesting some batches function better than others. Additionally, supplements are not required to third-party test

products for purity and content, which allows for the possibility that non-listed ingredients are included and ingredients that are purportedly included are missing. Such inconsistencies would likely inflate the variability of experimental measurement, contributing to inconsistencies in the reported effects of dietary supplements. Perhaps the most significant limitation to commercially available dietary supplements is the near-complete absence of scholarly evidence supporting individual product safety and efficacy. Another interesting and significant variable worth discussing is the matter of funding for research on dietary supplements. Multiple articles disclose that funding is contributed by the companies which market the product for retail purposes. This is not surprising because cost of biological research continues to increase while funding opportunities remain limited; therefore it is logical that select companies have outsourced primary research opportunities to the university setting. Surprisingly, many dietary supplements advertise one or multiple non-biased studies illustrating efficacy of their product, however often times these studies have not been systematically peer-reviewed or readily shared with the scientific community making the original research difficult to locate. In addition, identification and tracking of adverse events with the majority of studies would not meet requirements for similar pharmaceutical agents [6]. Of the known adverse effects, dry mouth, insomnia, nervousness, palpitations, headache hypertension, arrhythmias, myocardial infarction, stroke, and seizures have been linked to consumption of ingredients such as caffeine and ephedra, suggesting a major need for further safety evaluation prior to consumption [6, 7]. Lastly, along with limited evidence of whole-body efficacy and safety, there is inadequate evaluation of the molecular effects of commercially available dietary supplements. Our lab previously demonstrated that select dietary supplements containing a variety of ingredients could effectively increase skeletal

muscle metabolism and mitochondrial content *in vitro*, however additional information is required to adequately describe cell- and tissue-specific effects of thermogenic products [45, 46].

Concluding Remarks

Increasing obesity prevalence and consumer interest in thermogenic dietary supplements support the need for further research of these and other supplement ingredients. From the available evidence, it appears that commercially available dietary supplements advertised to stimulate metabolism have the propensity to increase metabolic rate. Despite significant increases in resting energy expenditure, it is doubtful that commercially available thermogenic products stimulate metabolism more than consumption of food products containing equivocal content of caffeine/stimulants and/or polyphenols. Moreover, it should be mentioned that increases in metabolism induced by food or dietary supplement are small, contributing only subtly to metabolic rate. Additional research is necessary to identify the precise mechanisms by which commonly consumed ingredients function to alter energy expenditure and what corresponding molecular adaptations may develop.

Conflict of Interest

The authors declare no conflict of interest. No funding was received for this work.

Abbreviations

AC - Adenylate Cyclase

AMP - Adenosine Monophosphate

AMPK - 5'Adenosine Monophosphate-Activated Protein Kinase

ATP - Adenosine Triphosphate

cAMP - 3,5-cyclic-Adenosine Monophosphate

COMT - Catechol O-methyltransferase - COMT

CREB - cAMP-Related Element Binding Protein

EGCG - Epigallocatechin Gallate

EGC - Epigallocatechin

ECG - Epicatechin gallate

EC - Epicatechin

G-CPR - G-Coupled Protein Recepetor

PG - Prostaglandin

PKA - Protein Kinase A

NE - Nor-Epinepherine

NRF - Nuclear Respiratory Factors

PGC-1α - Peroxisome Proliferator-Activated Receptor γ Coactivator-1α

PPARα - Peroxisome Proliferator-Activated Receptor α

PPARγ- Peroxisome Proliferator-Activated Receptor γ

PDE - Phosphodiesterase

SREBP-1c - Sterol Regulatory Element Binding Protein-1c

UCP3 - Mitochondrial Uncoupling Protein-3

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Chapter 2

Dietary Stimulators of the PGC-1 Superfamily and Mitochondrial Biosynthesis in Skeletal Muscle. A Mini-Review

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The final publication of this article is available at:

http://link.springer.com/article/10.1007%2Fs13105-013-0301-4

Abstract

Mitochondrial dysfunction has been linked to many diseases including metabolic diseases such as diabetes. Peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1) is a superfamily of transcriptional co-activators which are important precursors to mitochondrial biosynthesis found in most cells including skeletal muscle. The PGC-1 superfamily consists of three variants all of which are directly involved in controlling metabolic gene expression including those regulating fatty acid oxidation and mitochondrial proteins. In contrast to previous reviews on PGC-1, this mini-review summarizes the current knowledge of many known dietary stimulators of PGC-1 and the subsequent mitochondrial biosynthesis with associated metabolic benefit in skeletal muscle.

Key Words: Caffeine; Phytochemicals; 5' adenosine monophosphate-activated protein kinase (AMPK); Nuclear Respiratory Factors 1 and 2 (NRF1/2); Estrogen Related Receptor (ERR); Mitochondrial Transcription Factor A (TFAM)

Abbreviations

AC- Adenylate Cyclase

AICAR- 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

AMPK- 5' adenosine monophosphate-activated protein kinase

ATF- Activating Transcription Factor

CAMK- Calcium/Calmodulin Dependent Protein Kinase

CREB- cAMP Related Element Binding Protein

DHA- Docosahexaenoic Acid

EPA- Eicosapentaenoic Acid

ERR- Estrogen Related Receptor

FOXO- Forkhead Box Proteins

GCN5- General Control Nonderepressible 5

GPCR- G-Protein Coupled Receptors

HIF- Hypoxia Inducible Factor

MAPK- p38 Mitogen-Activated Protein Kinase

MEF-2- Myocyte Enhancing Factor

MyoD- Myogenic Regulatory Factor D

NO- Nitric Oxide

NRF 1/2- Nuclear Respiratory Factors 1 and 2

PGC-1α- Peroxisome Proliferator-Activated Receptor γ Co-activator-1

PGC-1- PGC Superfamily

PDE- Phosphodiesterase

PKA- Protein Kinase A

PPAR- Peroxisome Proliferator-Activated Receptor

PUFA- Poly-Unsaturated Fatty Acids

RXR- Retinoid X Receptor

SIRT- Sirtuin

SRC3- Steroid Receptor Coactivator 3

TFAM- Mitochondrial Transcription Factor A

TFBs- Transcription Specificity Factors TFB1M and TFB2M

TTA-Tetradecylthioacetic Acid

TZD- Thiozolidinediones

VEGF- Vascular Endothelial Growth Factor

Introduction

Obesity and related metabolic disorders are increasingly recognized as major contributors to preventable death and increasing healthcare cost. It is forecasted that by 2030 over 85% of adult Americans will be overweight, over 50% of whom will be clinically obese which is associated with the development of several comorbidities including cancer, diabetes, hypertension, cardiovascular disease, and premature death [97, 116]. Following a prudent diet by restricting energy intake and increasing energy expenditure through physical activity are two prominent behavioral modifications used to combat obesity and related disorders [25, 97]. Despite the consistent efficacy of diet and exercise to achieve weight-loss, many fail to maintain such lifestyle changes and are unable to maintain ideal or healthy body weights [25, 97]. Due to the unsustainable nature for many people of behavioral modifications, patients and clinicians alike are interested in functional chemicals in dietary components, dietary supplements and pharmaceuticals to aid in long-term weight loss. Pharmaceuticals have well established efficacy and safety data, however increasing obesity incidence suggests that available interventions are insufficient to overcome obesity promoting factors, such as sedentary lifestyles and chronic excess energy intake. Functional secondary metabolites found in food and dietary supplements have emerged as a source of great consumer interest; however, such metabolites generally lack evidence validating their safety or efficacy for weight loss. In addition to purported metabolic benefits, many dietary supplements and food metabolites are advertised to increase athletic performance.

Skeletal muscle is meaningful for whole-body energy balance because skeletal muscle contributes a large proportion of overall metabolic rate. Uniquely, skeletal muscle has the ability to quickly adapt its metabolism in response to available metabolites and to regular exercise. The beneficial metabolic adaptations in skeletal muscle in response to exercise are largely due to the

change in mitochondrial size and content. Increases in muscle mitochondrial content are associated with heightened fatty acid oxidative enzymes, citrate cycle enzymes, and increased respiratory chain components, all of which contribute to an augmented ability to generate ATP. More mitochondrial proteins and total mitochondrial content increase the capacity to synthesize ATP, thereby creating a favorable cellular environment to enhance both total body fat oxidation and/or enhance athletic performance. In order to increase muscle mitochondrial content, a protein known as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) must be activated along with several other intracellular signaling molecules. Low levels of PGC-1α have previously been linked to obesity, diabetes, and several other metabolic disorders [60, 97]. Excess energy intake combined with sedentary lifestyle lead to mitochondrial dysfunction and suppressed PGC-1α expression which play a significant role in the development and prognosis of obesity, diabetes, and other metabolic disease [67, 68]. In addition, pharmaceutical stimulators of PGC-1 such as metformin have implications for weight loss in overweight and obese subjects, people who are likely to require medicinal intervention for co-morbidities such as diabetes [36, 93]. Because several pharmacological agents commonly prescribed are effective stimulators of the PGC-1 pathway but do not cause rapid weight loss, it is clear that a single PGC-1 stimulator will not be a "cure" for obesity. However, because of the potent effects of PGC-1 stimulation on metabolic characteristics (such as muscle fiber type), it is also conceivable that dietary and pharmacological PGC-1 stimulators represent another tool in the battle against rising obesity prevalence. Perhaps the most promising evidence is demonstrated by the effects of exercise and energy restriction (common techniques for weight loss seekers) on metabolic disease can be linked to stimulation of PGC-1α [43, 75]. This work outlines much of the current knowledge of dietary metabolic stimulators of PGC-1α expression which preludes increases in cellular

oxidative capacity. This review provides framework for future research in establishing potential natural products and food ingredients which may elicit metabolic benefits similar to exercise and energy restriction, and may assist in current therapies for metabolic disease.

Mitochondrial Biosynthesis

Mitochondrial biosynthesis is initiated with increased transcription of both nuclear and mitochondrial DNA (mtDNA; which consists of 13 genes) [35]. The process is predominantly regulated by nuclear gene expression of mitochondrial transcription factor A (TFAM) which regulates mitochondrial gene expression through direct interaction with the mitochondrial genome along with mitochondrial transcription specificity factors TFB1M and TFB2M [35]. Specifically, TFAM expressed in the nucleus is responsible for synthesis of mitochondrial electron transport chain proteins that are downstream targets of the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) [26, 89, 114]. NRF1/2 promotes mitochondrial biosynthesis through induction of TFAM resulting in both mtDNA replication and mtRNA expression as well as induction of TFBs [35, 38]. Additionally, interactions with a series of nuclear factors including estrogen related receptor (ERR), retinoid X receptor (RXR) and cAMP related element binding protein (CREB) allow NRF1/2 to directly control metabolic, angiogenic, myofibrillar, and calcium handling pathways in skeletal muscle [82]. Regulation of NRF1/2 activity, along with subsequent TFAM expression is coordinated by one of the most heavily researched co-activators of metabolism known as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which requires induction and/or activation via phosphorylation or deacetylation, described in detail below [35, 45, 90, 127]. PGC-1α is a transcriptional coactivator that is essential for mitochondrial biosynthesis and activates genes that regulate energy homeostasis and metabolism [50, 122, 123]. In addition to stimulating mitochondrial biogenesis through nuclear NRF1/2 expression, PGC-1α induces genes responsible for fatty acid oxidation through increased

peroxisome proliferator-activated receptor alpha (PPARα) expression, which complements the corresponding increases in respiratory chain components [80, 112, 125].

Additionally, PGC-1α interacts with PPAR and retinoid X receptor (RXR), both of which must be bound to their respective ligands. Ligand-bound PPAR and RXR form a heterodimer which interacts with the PPAR response element (PPRE) located on the promoter regions of nuclear genes encoding for mitochondrial transcription factors and fatty acid oxidation [12, 66, 121, 123]. PPAR can be sequestered by the liver X receptor (LXR), or LXR can competitively interact with RXR inducing sterol receptor element binding protein (SREBP) thereby increasing fatty acid synthesis and suppressing PPAR activity thus diminishing fatty acid oxidation.

PGC-1α Induction

PGC-1α is functionally similar to PGC-1β and PGC-1-related coactivator (PRC) which comprise the PGC-1 superfamily (PGC-1), acting to synergistically coordinate mitochondrial biosynthesis and energetic adaptations [106]. PGC-1α is the most heavily researched and best characterized [106]. PGC-1α is a combination of co-activators consisting of three variants (PGC-1α -a, PGC-1α -b and PGC-1α -c) with tissue-specific levels of expression [97, 102, 103]. When inactive, PGC-1α resides in the cytosol where it is hyper-acetylated by general control nonderepressible 5 (GCN5) or steroid receptor coactivator 3 (SRC3) which is promoted by energy excess and diets rich in fat [8]. PGC-1α can be deacetylated through interactions with SIRT1, allowing PGC-1α to translocate to the nucleus with or without phosphorylation, but PGC-1α must be phosphorylated in order to be active [3, 6, 8, 53]. PGC-1α is phosphorylated in response to numerous stimuli, most of which include signals of increased energy demand. Specifically, increasing ATP consumption and increasing cAMP formation results in the formation and activation of 5' adenosine monophosphate-activated protein kinase (AMPK) which can directly phosphorylate

PGC-1α. Additionally, heightened AMPK activity stimulated via exercise or energy restriction promotes PGC-1α transcription through hyperphosphorylation of Forkhead Box Protein 3 (FOXO3) and through phosphorylation of cAMP response element-binding (CREB) proteins which binds to and activates the cAMP response element (CRE) site on the PGC-1α promoter and drives PGC-1α expression [8, 75, 121], Furthermore, although not shown in Figure 1, exercise and energy restriction cause a rapid activation of p38 mitogen-activated protein kinase (MAPK) which works in coordination with AMPK to promote AMPK-phosphorylation of PGC-1α leading to PGC-1α activation and increased PGC-1α expression [34, 45, 46, 51, 81]. MAPK also mediates the increase in PGC-1α expression by phosphorylating activating transcription factor (ATF-2), a member of the CREB proteins and myocyte enhancing factor (MEF-2) which bind the PGC-1α promoter further driving PGC-1α expression [39, 121]. Similar to changes in energy status, increased release of Ca^{2+} from the sarcoplasmic reticulum as seen during repeated muscle contraction induces increases in mitochondrial biogenesis [71-73, 97, 122]. Cytosolic Ca^{2+}-activates the enzymes calcium/calmodulin dependent protein kinase (CaMK) and calcineurin which increases PGC-1α expression through CREB phosporylation [39, 52, 58, 87, 91, 97, 122]. Moreover, increased cytosolic Ca^{2+} leads to expression of PGC-1 through activation of p38 MAPK [120]. PGC-1α can also act to drive its own expression through interactions with SIRT1 and myogenic regulatory factor (MyoD) [3, 8]. Once activated, an auto-stimulatory loop causes PGC-1 to further increases its own expression [39, 97]. Figure 1 illustrates the activation and induction of PGC-1α along with corresponding interactions which promote mitochondrial biosynthesis and increases in fatty acid oxidation components.

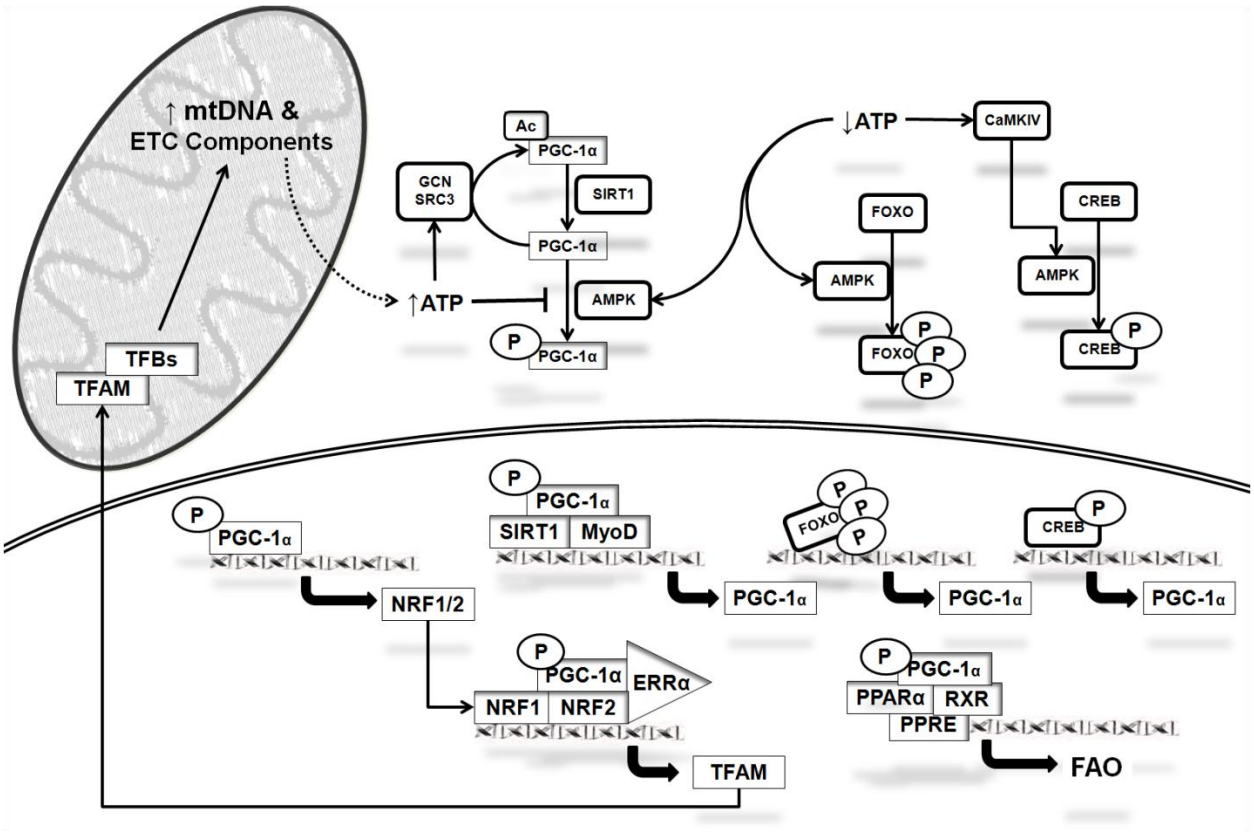

Fig 2 Process of PGC-1α activation, induction, and downstream effects leading to mitochondrial biosynthesis and fatty acid oxidation

Stimulation of PGC-1 Activation

PGC-1 Stimulation

It is important to mention the vast effects that behavior can have on metabolic capacities of skeletal muscle, such as PGC-1 expression. Exercise has multiple effects on PGC-1 α, which have numerous subsequent effects that increase mitochondrial function and metabolic capacities [106]. Additionally, fasting has been shown to enhance PGC-1 expression and increase oxidative capacities through AMPK activation [11]. Prolonged exercise or fasting/energy restriction increases concentrations of free fatty acids (FFA) which increase the expression of the transcription factor PPARδ in muscle and results in an increase in mitochondria [117]. Activation of the PGC-1 superfamily by exercise and fasting is in part a result of hormone release in response to altered energy homeostasis. Exercise stimulation of β-2 adrenoceptor activation by catecholamines results in increased cAMP thereby activating CREB transcription factors that bind to the PGC-1α promoter increasing PGC-1α expression [65, 97].

Pharmacological, Dietary, Behavioral Stimulators of PGC-1

Pharmacological PGC-1 Stimulators

The compound, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is commonly used as an activator of AMPK which leads to PGC-1α activation through phosphorylation, which appears to be necessary for synthesis of mitochondrial proteins and GLUT4 expression [56]. AICAR is taken up by muscle cells and converted to the AMP analog ZMP which activates AMPK and results in increased mitochondrial biogenesis through PGC-1α activation [28, 47, 56, 61, 103, 119]. Activation of the AMPK pathway with the chemical GW501516 also increases mitochondrial biogenesis in skeletal muscle cells [5, 16]. Additionally, GW501516 acts to increase PPARα expression and DNA binding leading to increased oxidative capacity which is reduced in PGC-1α knockout rodents [5, 49]. Research-grade chemical uncouplers of oxidative

phosphorylation such as 2,4-dinitrophenol (DNP) and *p*-trifluromethoxy phenylhydrazone (FCCP) have been shown to induce PGC-1α/β, NRF-1, TFAM, and mitochondrial protein expression through reduction of ATP levels in fibroblasts [85]. Recently, our lab identified that treatment with DNP induces PGC-1α, and increases both metabolic rate and mitochondrial content in muscle cells and increased total glycolytic and oxidative metabolism [110].

Metformin, an anti-diabetic biguanide which inhibits hepatic gluconeogenesis, inhibits mitochondrial complex I thereby increasing the AMP:ATP ratio stimulating AMPK phosphorylation [113]. Metformin-induced AMPK phosphorylation in skeletal muscle increases PGC-1α, mitochondrial oxidative enzymes, and cytochrome C expression ultimately amplifying mitochondrial function [101]. Other agents such as β-2 adrenoceptor agonists such as clenbuterol have been shown to increase intracellular cAMP and phosphorylated AMPK leading to enhanced PGC-1α expression [37, 60, 97]. Similarly, thiozolidinediones such as Pioglitazone and Rosiglitazone act as direct ligands for PPARα/γ promoting activation of PPRE, which acts with PGC-1α as a coactivator to drive gene expression [83]. Recently, it was demonstrated that while thiozolidinediones act as ligands for PPARs, they also act through cyclin-dependent kinase 5 (CDK5) to activate gene activity [13]. Treatment of C2C12 mouse myocytes with a Pioglitazone has been shown to reverse insulin insensitivity and malfunctioning energetics in part through induction of PGC-1α [74]. In therapy-naive humans, pioglitazone has been shown to increase PGC-1α expression through AMPK phosphorylation [15]. In addition, loss of mitochondrial function and PGC-1α expression can be restored in obese ZDF rats treated with troglitazone [48]. In humans, the effect of rosiglitazone is likely a product of muscular oxidative capacity controlled through PGC-1 α, although metabolic adaptations may occur independent of mitochondrial biogenesis [63]. Fibrates, used clinically to reduce triglycerides act as synthetic

ligands for PPARα, as well as by increasing lipoprotein lipase leading to increased fat oxidation and decreased fat biosynthesis [96]. Benzafibrate was shown to act on skeletal muscle inducing PGC-1α gene expression in both cardiac and skeletal muscles from female mice and cultured myocytes, functioning in a PPARδ-mediated fashion [44]. Table 1 summarizes some of the pharmacological strategies used to increase PGC-1 and related mitochondrial content.

Table 2 Summary of pharmacological stimulators of PGC-1 and mitochondrial biosynthesis in various experimental models of skeletal muscle

Mitochondrial Stimulators	Experiment Model	Findings
AICAR	Primary rat skeletal muscle	↑ PGC-1 mRNA [28, 30]
	Mice (WT/PGC-1 KO)	↑ PGC-1, phospho-AMPK, GLUT4 transporter mRNA and protein [56]
	L6 Myotubes	↑ CaMK, AMPK, COX1/4, PGC-1α protein [61]
	Mice (WT/ Mutant AMPK-DN)	↑ PGC-1 isoform mRNA [103]
	Rat skeletal muscle	↑ Mitochondrial enzymes and protein [119]
Metformin	Wistar rat skeletal muscle	↑ PGC-1α and phospho-AMPK protein [101]
GW501516	Mice with varied fat diets	↑ PGC-1α mRNA, phospho-AMPK and phospho-ERK protein and AMP:ATP [5, 16]
FCCP/DNP	Fibroblasts and mice	↑ PGC-1 mRNA and protein, mitochondrial proteins, NRF, AMPK, CREB [85]
	Human rhabdomyosarcoma cells	↑ PGC-1 mRNA and protein, mitochondrial proteins, glycolytic/oxidative metabolism [110]
β-2 adrenoceptor agonists	Wistar rat muscle and C57/BL6 male mice	↑ PGC-1 mRNA, phospho-Akt, cAMP [37]
Rosiglitazone (TZD)	C2C12 Mouse Myoblasts & db/db mice	↑ PGC-1 mRNA /protein, mitochondrial mass [74]
	10 obese and 10 healthy subjects	↑ PGC-1, PPARβ/δ mRNA [63]
Pioglitazone (TZD)	26 drug-naïve patients	↑ PGC-1α/β, phospho-AMPK protein, and PPARα mRNA [15]
Fibrates	Mouse skeletal/cardiac muscle and C2C12/L6 myocytes	↑ PGC-1α, PPARβ/δ mRNA, PPRE activity [96]

Dietary PGC-1 Stimulators

Unlike synthetic chemicals, which often possess significant toxicity and lethality, many dietary constituents offer similar benefits with greatly reduced side effects. Commonly consumed central nervous stimulants such as caffeine increase AMPK phosphorylation, as well as cAMP accumulation which activates CREB ultimately leading to PGC-1 expression [19, 52, 61, 71, 72]. Caffeine also works by increasing intracellular Ca^{2+} leading to CaMK activation and subsequent AMPK phosphorylation [19, 52, 61, 71, 72]. We previously demonstrated that both research grade caffeine and dietary supplements containing stimulants can effectively induce PGC-1 resulting in greater mitochondrial content and oxidative capacity in skeletal muscle cells [110, 111]. Limited information regarding other dietary methyl-xanthines such as theophylline and theobromine (found in tea and chocolate, respectively) have been shown to increase skeletal muscle cAMP, one precursor to PGC-1 expression [55]. Additionally, selected xanthine metabolites can increase calcium uptake and reduce cellular ATP content in rat-liver which are known cellular precursors to increased PGC-1 expression leading to mitochondrial biosynthesis [20]. Forskolin (found in the herb *Coleus forskohlii*), a common constituent of many over-the-counter dietary supplements is also proposed to function through a related mechanism by increasing mitochondrial enzyme activity [18, 55]. Similar to caffeine, catecholamines (such as epinephrine) which are released during intense exercise have been shown to increase PGC-1 expression in mouse and rat skeletal muscle and adipocytes [30, 100, 103]. β-2 adrenoceptor agonists such as synephrine and ephedra also promote the formation of cAMP [23], although there appears to be no direct evidence that either catecholamine-like compound increases PGC-1 expression or mitochondrial content. Green tea and black tea extracts are rich in polyphenols such as epigallocatechin gallate (EGCG), which are proposed to increase effects of circulating catecholamines through inhibition of catechin-O-methyl transferase [24, 42]. EGCG has been

shown to significantly increase GLUT4 translocation in skeletal muscle of rats, as well as increase glucose uptake and GLUT4 translocation in L6 myotubes [105]. Moreover, in high fat-fed C57bl/6J mice, EGCG increased mRNA levels of several mitochondrial biosynthetic genes including NRF-1 and UCP3 (downstream targets of PGC-1), as well as PPARα [88]. EGCG was also shown to increase AMPK and p38MAPK phosphorylation which promotes PGC-1 phosphorylation and activation [21].

Poly-unsaturated fatty acids (PUFAs) are of great interest for metabolic and inflammatory disease. Longer chain omega 3 fatty acids appear to stimulate PPRE activation by acting as direct ligands for PPAR, thereby increasing association of RXR-PPAR leading to increased fatty acid oxidation with decreased fatty acid biosynthesis [17, 96]. Eicosapentaenoic acid (EPA) and tetradecylthioacetic acid (TTA) were shown to have a pronounced effect on PGC-1 expression, fatty acid oxidation, and rodent body weight and composition [27, 59]. Male C57BL6 mice fed a diet rich in fish oil (3.4% energy) display increased PPARα/γ expression along with PGC-1α, NRF, and TFAM ultimately leading to increased mitochondrial mass and number in [54]. Additionally, combination EPA/DHA (docosahexaenoic acid) has been shown to reduce fat mass in C57BL/6J mice, and DHA independently induced NRF-1 expression in a PGC-1 dependent manner in 3T3-L1 adipocytes [27]. Moreover, we have recently shown that combination EPA/DHA can increase mitochondrial content and function [109]. In addition to long-chain fatty acids such as EPA and DHA, short chain fats such as butyric acid (found in cheese and butter) have been shown to increase PGC-1 expression, as well as increase oxidative capacities in L6 myocytes and skeletal muscle of rodents [33].

Similar to select lipids and stimulants, the amino acid leucine has been shown to increase mitochondrial content in both adipocytes and muscle cells [9, 98, 99]. In muscle, leucine

increases SIRT1 phosphorylation, acting as a potent stimulator of both AMPK and PGC-1 activation [9, 98, 99]. It is speculated however, that the effects of leucine on protein synthesis and total energy expenditure also indirectly lead to activation of AMPK by increased consumption of ATP leading to increased mitochondrial content to support heightened energy consumption [108]. Our group recently verified the effects of leucine treatment on PGC-1 expression with simultaneous increase in mitochondrial content using human and mouse cell models [108]. Other nitrogen-containing compounds including arginine and arginine-like metabolites, increase nitric oxide (NO) which stimulates the formation of cGMP and cooperatively interacts with AMPK and CaMK leading to PGC-1 induction [31, 57]. In adipocytes, arginine was shown to increase mitochondrial content in ambient temperatures, and intensify cold-induced browning of adipocytes [77].

In addition to essential nutrients and amino acids, select phytochemicals (such as EGCG described above) have become of great interest for treating/preventing chronic disease, including diseases linked to mitochondrial malfunction. Resveratrol found in grapes, peanuts and most famously in red wine was thought to activate SIRT1, which deacetylates PGC-1 and promotes AMPK phosphorylation of PGC-1 and FOXO leading to PGC-1 activation and expression thereby improving insulin sensitivity and cellular antioxidant status [6, 8, 53, 126]. Recently, it has become unclear if resveratrol acts by directly activating SIRT or solely by activating AMPK through indirect means to drive PGC-1 activation and expression; however, experiments performed with SIRT1 knockout mice illustrate that resveratrol increases AMPK in a SIRT1 mediated fashion in skeletal muscle [6, 22, 78]. Resveratrol treatment appears to increase expression and deacetylation of PGC-1 leading to significantly elevated mitochondrial DNA and content which appears to function in part through AMPK activation and through increased SIRT

expression [53, 126]. In muscle-specific SIRT1 knockout mice, resveratrol can still function to increase mitochondria by activating existing PGC-1 protein [64]. In addition, supplementation with resveratrol has been shown to improve many clinically meaningful characteristics including circulating lipids, glucose, and inflammatory markers [104]. Despite some controversy regarding the exact mechanism(s) of action, resveratrol appears to be a potent stimulator of PGC-1 and mitochondrial biogenesis in skeletal muscle. Bruckbauer and colleagues evaluated the synergistic effect of simultaneous leucine and resveratrol treatment of cultures 3T3 adipocytes and C2C12 myocytes and found that concurrent treatment significantly elevated SIRT1 and SIRT3 in both cell models [10]. Additionally, they found that combination leucine and resveratrol increased AMPK content and fatty acid oxidation under both high and low glucose in cultured adipocytes [10].

Fucoxanthin, a chlorophyll-associated carotenoid component of chloroplasts found in sea weed has also recently been shown to increase GLUT4 translocation of skeletal muscle in rodents though activation of Akt and insulin receptor in part as a result of PGC-1 induction [69]. Lipoic acid and ubiquinol (CoQ10) have been shown to increase nuclear PGC-1 levels with heightened PPARγ activation, NRF-2, TFAM, and mitochondrial content in C2C12 mouse myoblasts [115]. Several experiments have shown that statin treatment reduces CoQ10 levels resulting in mitochondrial dysfunction and possibly myopathy [1, 7]. Interestingly, our laboratory recently demonstrated that concurrent statin and CoQ10 treatment rescues statin-induced metabolic dysfunction in muscle cells [107]. Despite increasing interest in food chemicals and natural products, the effects of many of the aforementioned components on metabolic gene expression and molecular metabolism still remain unexplored. Although not an exhaustive list of all

potential dietary metabolic stimulators, Table 2 summarizes some evidence supporting dietary stimulation of PGC-1α and resulting mitochondrial biosynthesis.

Table 3 Summary of dietary stimulators of PGC-1 and mitochondrial biosynthesis in various experimental models of skeletal muscle

Mitochondrial Stimulators	Experiment Model	Findings
Caffeine	Primary rat myoblasts	↑ PGC-1 mRNA [52]
	Human rhabdomyosarcoma cells	↑ PGC-1 mRNA and protein, mitochondrial proteins, glycolytic/oxidative metabolism [110]
	L6 Myotubes	↑ CaMK, AMPK, COX1/4, PGC-1α protein [61]
	L6 Myotubes	↑ AMPK, COX-1, cytosolic Ca2+, mitochondrial enzymes, fatty acid oxidation↑PGC-1 protein, COX, CytC, NRF-1/2, tFAM cytosolic Ca^{2+} [71-73]
Xanthine Metabolites	Rat myotubes	↑ cAMP and mitochondrial enzymes/activity [55]
	Rat hepatocytes	↓ Mitchondrial ATP [20]
Forskolin	Rat myotubes	↑ cAMP and mitochondrial enzymes/activity [55]
	Human myotubes	↑ Mitochondrial content [18]
Catecholamines	Primary rat skeletal muscle	↑ PGC-1mRNA [30]
	Rat adipose tissue	↑ PGC-1 mRNA [100]
	Mice (WT/ Mutant α1-AMPK-DN)	Activates β-2 receptors, ↑PGC-1 isoform mRNA [103]
Green Tea (EGCG)	Rat muscle and L6 myotubes	↑ GLUT4 translocation [105]
	C57bl/6J mice	↑ NRF-1 UCP3, and PPARα mRNA [88]
	C2C12 myoblasts	↑ phospho-AMPK and phospho-38MAPK [21]
EPA/DHA	Mice adipose tissue	↑ PGC-1 and NRF-1 mRNA, mitochondrial proteins, fatty acid oxidation ↑ fatty acid oxidation, mitochondrial content, ↓ plasma TAGs [27]
	Male C57BL6 mice	↑ PPARα/γ, PGC-1α, NRF, and TFAM expression, and total mitochondrial mass [54]
	Human rhabdomyosarcoma cells	↑ PGC-1 mRNA and protein, mitochondrial proteins, glycolytic/oxidative metabolism [109]
Butyrate	C57BL/6 mice	↑ PGC-1 and AKT mRNA and protein, AMPK and p38 activity, muscle fiber type [33]
Leucine	C2C12 myoblasts and adipocytes	↑ PGC-1 and NRF mRNA and protein, mitochondrial proteins, mitochondrial staining, oxidative metabolism [98, 99]
	C2C12 myoblasts	↑ SIRT activation, ↑NRF and UCP RNA [9]
	Human rhabdomyosarcoma cells & C2C12 myoblasts	↑ PGC-1 and cytochrome C protein, mitochondrial proteins, glycolytic/oxidative metabolism with altered ATP production [108]
Arginine/Nitric Oxide Donors	C2C12 myoblasts and L6	↑ AMPK protein, ↑ PGC-1 mRNA [57]
	C2C12 myoblasts and L6	↑ cGMP, AMPK, and CaMK [77]
	Zucker rats	↑ AMPK, CaMK, PGC-1 mRNA in adipose [31]
Resveratrol	C57Bl/6J and KKAy mice	↑ PGC-1, ERR, SIRT, oxygen consumption [6, 53]
	Catch-up growth mice	Rescued loss mitochondrial function and PGC-1 expression [126]
	Adult-Inducible SIRT1 Knockout Mice	↑ PGC-1α/β, NRF, TFB, TFAM, AMPK, and SIRT expression with ↑ mitochondrial content through multiple mechanism dependent on dose [78]
	Muscle-specific SIRT-knockout mice and C2C12 myoblasts	↑ PGC-1α, phospho-AMPK, and SIRT expression with ↑PGC-1α activity and ↑ mitochondrial content [64]
	10 Healthy obese males	↑ PGC-1 and phospho-AMPK protein [104]
Fucoxanthin	KKAy mice	↑ PGC-1 and AKT phosphorylation [69]
Lipoic Acid/CoQ10	C2C12 myoblasts	↑ PGC-1, TFAM and NRF mRNA [115]

Downstream PGC-1α Effects

As previously mentioned, PGC-1α modifies metabolic rate and expression of genes involved in metabolism, fat oxidation and mitochondrial biosynthesis [80, 112, 125]. PGC-1α is also an important signaling molecule in the activation and regulation of gluconeogenesis, which is likely mediated through FOXO1 and ERR-α [2, 41, 84, 95, 125]. Further evidence suggests that an increase in PGC-1α can improve insulin sensitivity and improve muscle function. It has also been identified that PGC-1α is essential for the recovery from the diminished ATP caused by chemical uncoupling as evidenced by the lack of recovery in PGC-1α null cells and animals [85].

PGC-1α induction and mitochondrial biosynthesis are usually stimulated by heightened energy needs which require increased nutrient uptake. Enhanced expression of PGC-1β leads to corresponding increases in components that facilitate fatty acid oxidation including carnitine acyl transferase and fatty-acyl dehydrogenases, demonstrated in C2C12 myocytes [29, 32]. In addition to increases in fatty acid oxidative components, PGC-1 also increases expression of GLUT transporters namely GLUT4 to facilitate increased glucose uptake and metabolism [56, 109]. PGC-1 induction can also increase expression of mitochondrial uncoupling proteins which enhance total cellular energy expenditure [29]. Ectopic PGC-1 expression has also been linked to increased skeletal muscle angiogenesis through induction of VEGF via ERR activation independent of HIF in skeletal muscle myocytes and *in vivo* [86]. These observations are contrary to previous hypotheses that PGC-1 functions to control oxygen availability through a coupling with HIF-1 [4, 70]. This information supports the hypothesis that chronic PGC-1 activation leads to increased oxidative capacity through heightened oxygen availability which supports increases in mitochondria and oxidative components. Figure 2 summarizes many previously mentioned dietary stimulators of mitochondrial biosynthesis with proposed mechanisms of action and several downstream effects.

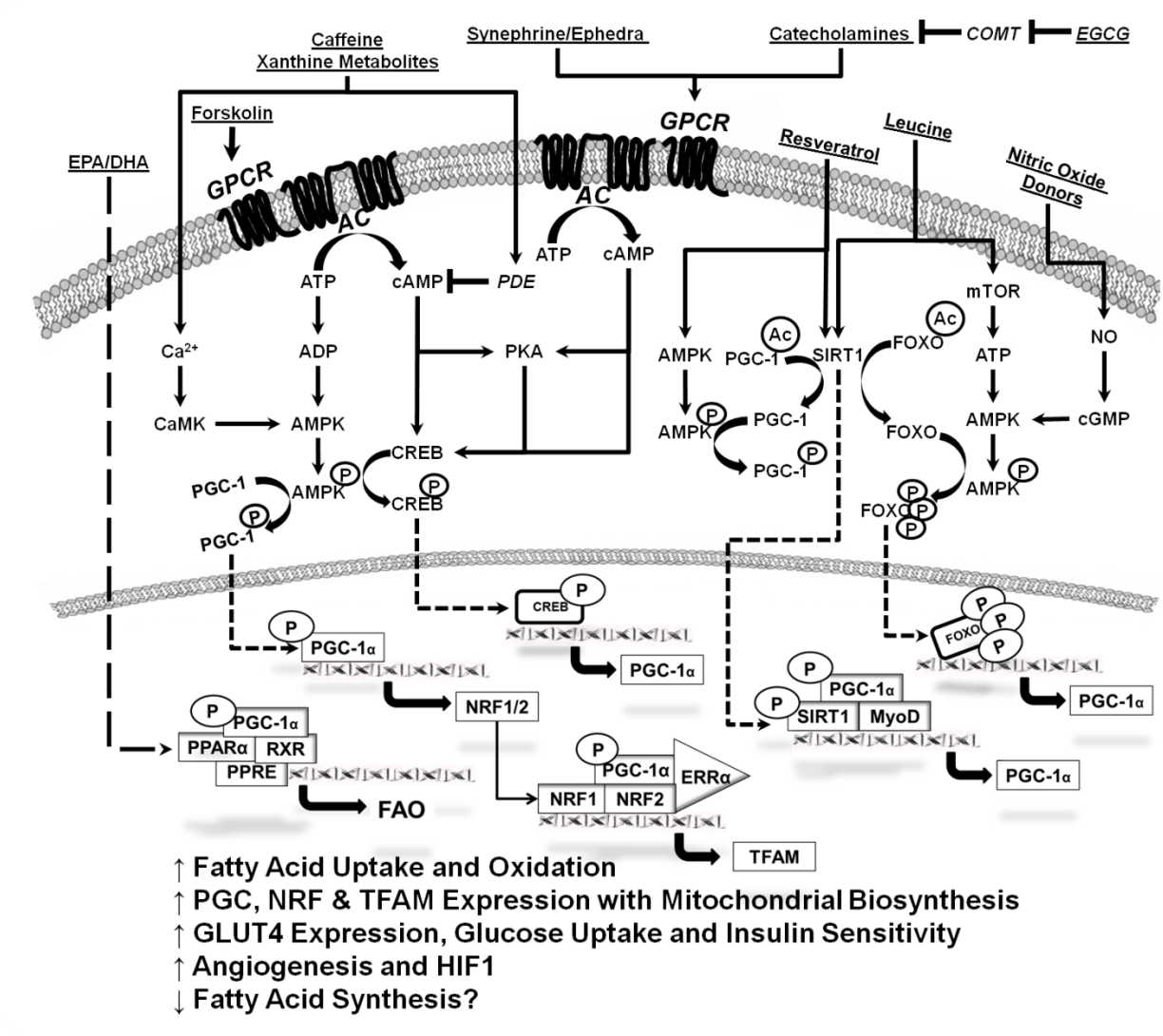

Fig 3 Summary of documented dietary stimulators of PGC-1 and related nuclear factors leading to increased mitochondrial biosynthesis and other metabolic events

Notes: short dashed line indicates mobilization to the nucleus leading to altered metabolic gene expression. Long dashed line indicates poly-unsaturated fats act as ligands for the PPAR/RXR heterodimer.

Concluding Remarks

Clinically, the relationship between low PGC-1α expression and several metabolic diseases including type II diabetes/obesity and neurological disease has been identified [14, 76, 79, 94, 124]. Patients with early onset of type II diabetes express constitutively lower levels of skeletal muscle PGC-1α [40]. Low PGC-1α is also associated with reduced expression of oxidative phosphorylation genes, decreasing fatty acid oxidation and energy utilization [66, 76, 92]. These observations and others support the hypothesis that mitochondrial biogenesis mediated through pharmacological or metabolic induction of the PGC-1α pathway is likely to be an effective therapeutic intervention for many disorders [118].

Current research has outlined many dietary factors that can increase both PGC-1α expression and the resulting increase in oxidative capacity. Much of the current literature is somewhat limited as a result of inferred increases in mitochondrial content/activity rather than direct measurement. In much of the preliminary work, only PGC-1 or related protein (or RNA) was measured and increased mitochondrial content was then inferred as an inevitable downstream effect. Improved understanding of energy dynamics combined with advances in technology has led to substantial improvements in current methods for quantifying mitochondrial biosynthesis (techniques which are reviewed elsewhere) [62]. Additionally, little is known about the translation from *in vitro* and animal models to therapeutic interventions in humans. Several questions come to mind. Are experimental doses practical and achievable from food or supplement? Is the functional food chemical altered once absorbed through the gut, and if so, how many functional analogs may exists? Will combinations of PGC-1 stimulators act synergistically, in a saturable way, or oppose one another? Further investigation is required to answer these, and many other questions pertaining to the potential efficacy of dietary PGC-1 stimulator for metabolic disease. Figure 2

and Table 2 describes the significant findings and conclusions of the literature summarized in this review.

Conflict of Interest
No funding was received for this work. The authors of this work declare no conflict of interest.

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Chapter 3

Effect of Novel Dietary Supplement on Metabolism *In Vitro* and *In Vivo*

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Abstract

Background: Obesity is an increasingly prevalent and preventable morbidity with multiple behavioral, surgical and pharmacological interventions currently available. Commercially available dietary supplements are often advertised to stimulate metabolism and cause rapid weight and/or fat loss, although few well controlled studies have demonstrated such effects. Our work uniquely describes the effects of a commercially available dietary supplement on resting metabolic rate in humans as well as the metabolic and biochemical effects *in vitro*. **Methods**: Human rhabdomyosarcoma cells (RD) and mouse myoblasts (C2C12) were cultured under standard conditions and treated with various doses of a commercially available supplement (RF) for various durations and assessed for changes in metabolism, metabolic gene expression and mitochondrial content. Additionally, human subjects ingested either placebo or RF in a double-blind placebo controlled fashion and metabolic rate and blood pressure were measured at 3 time points for 3 hours post-ingestion. **Results**: RF enhanced metabolism, metabolic gene expression, and mitochondrial content in both cell models. RF also enhanced energy expenditure in human male subjects without altering substrate utilization. RF also significantly increased systolic blood pressure. **Conclusion**: RF appears to increase metabolism immediately following ingestion, although additional research is needed to examine safety and efficacy for human weight loss.

Key Words: Obesity, Fat Burners, Resting Energy Expenditure, Mitochondrial Biogenesis

Background

Obesity is an increasingly prevalent morbidity with nearly two thirds of adult Americans overweight, and over 32% of men and 35% of adult women clinically obese [1]. It is forecasted that roughly 85% of adult Americans will be overweight, over half of which will be clinically obese by 2030 [2, 3]. Over the past decade, chemical and behavioral interventions that favorably modify metabolic rate have been central to obesity research. Several over-the-counter dietary supplements claim to increase metabolic rate and enhance fatty acid catabolism. Of the available over-the-counter dietary supplements, Ripped Freak® (RF) produced by PharmaFreak is purported to increase metabolic rate and fat metabolism.

Caffeine has been documented to increase metabolic rate in humans and in cell culture [4-10]. Human data suggests that caffeine elicits a dose-dependent increase in resting metabolic rate [9]. In addition, dietary components (such as green tea) have been shown to increase metabolism more effectively than caffeine alone [4]. Dietary supplements similar to RF were previously shown to increase markers of fat mobilization, metabolic rate (measured via indirect calorimetry), and reduce bodyweight and body fat (estimated via Dual-energy X-ray absorptiometry) in healthy young subjects following ingestion [11-18]. Our laboratory recently identified that treatment of cultured skeletal muscle with caffeine can increase both metabolic rate and mitochondrial content in muscle cells suggesting that commercially available metabolic stimulators might have similar effects [8]. Caffeine is believed to work through phosphodiesterase inhibition leading to increases in cAMP or through increasing cytosolic Ca^{2+} [8, 19, 20]. In addition, our lab recently showed that treatment with either of two similar over-the-counter supplements, or several other dietary components lead to increased metabolic rate and mitochondrial content in skeletal muscle cells [8, 21-24]. We demonstrated along with others

that stimulation of metabolism by dietary components induces many molecular adaptations including metabolic gene expression. Specifically, expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is increased following administration of various dietary stimulators of metabolism [8, 22-24]. PGC-1α acts as a transcriptional coactivator that is essential for mitochondrial biosynthesis and acts as a master regulator of energy homeostasis and metabolism [25-27]. Our group has previously reviewed the potential role for dietary components in the stimulation of PGC-1α and corresponding favorable metabolic adaptations for benefit in metabolic disease [28].

RF is a newer dietary supplement which has limited research regarding safety or efficacy. RF is advertised to increase metabolic rate, oxygen consumption, and fatty acid oxidation. RF is also purported to modify signal transduction and induction of genes that control energy homeostasis. Several of the ingredients purportedly comprising RF's proprietary blend including caffeine, green tea, raspberry ketones, and capsaicin have been previously linked to increased metabolic rate (Table 1). These ingredients are regularly promoted by vendors and figureheads of mainstream media, often without any supporting evidence of efficacy. Moreover, because supplements commonly contain a variety of ingredients in proprietary blend forms, and few controlled studies have been performed to address the metabolic effects at the cellular level, further work is needed to identify possible metabolic effects. This work seeks to evaluate the short term effects of RF treatment on metabolic characteristics in cultured muscle cells. Additionally, we investigated the effects of RF consumption on resting metabolic rate in healthy male volunteers.

Table 4 Purported composition of Ripped Freak®. *percent daily value not established

RIPPED FREAK® FORMULA 766.6 mg *
FAT BURNING METABOLISM AMPLIFIER 400mg *
Methyl EGCG™ (EGCG Derivative Stack) (Green Tea Extract/*Camellia Sinensis*)
-Epigallocatechin-3-O-(3-O-Methyl) gallate Ester (EGCG 3' 'Me)
-Epigallocatechin-3-O-(4-O-Methyl) gallate Ester (EGCG 4' 'Me)
-4'-O-Methyl-Epigallocatechin-3-O-Gallate Ester (EGCG 4'Me)
-Epigallocatechin-3-O-(3,4-O-Methyl) gallate Ester(EGCG 3' '4' 'diMe)
-4'-O-Methyl-Epigallocatechin-3-O-(4-O-Methyl) gallate Ester (EGCG 4'4' 'diMe)
Oleuropein Aglycone (Olive Leaf Extract/Olea Europaea)
1,3,7-Trimethyl-1H-Purine-2,6(3H,7H)-Dione Methyl Gallate Ester
(Caffeine) (Coffee/Coffea Arabica, Whole Bean)
UNCOUPLING PROTEIN / OXYGEN UTILIZATION AMPLIFIER 200mg *
CH-19 Sweet Red Pepper Ester Stack
(CH-19 Sweet Red Pepper Extract/Capsicum Annum, Fruit) (0.75% Capsiate)
4-Hydroxy-3-Methoxybenzyl (E)-8-Methyl-6-Nonenoate Ester
4-Hydroxy-3-Methoxybenzyl 8-Methyl-Nonanoate Ester
4-Hydroxy-3-Methoxybenzyl 7-Methyl-Octanoate Ester
HORMONE SENSITIVE LIPASE FAT MOBILIZATION AMPLIFIER 166.6mg *
4-(4-Hydroxyphenyl)-2-Methyl Ethyl Ketone (Raspberry Ketone)
4-(4-Hydroxyphenyl)-2-Butanone Methyl Gallate Ester (Raspberry Ketone - Gallic Acid)

Methods

Cell Culture- Human rhabdomyosarcoma cells (RD) and mouse myoblasts (C2C12) were purchased from ATCC (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4500mg/L glucose and supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 100U/mL penicillin/streptomycin, in a humidified 5% CO2 atmosphere at 37°C. Stock Ripped Freak® (RF) from PharmaFreak was purchased over the counter and was diluted to various concentrations in ethanol; doses for RD cells contained 200 µg/ml or 100 µg/ml and doses for C2C12 cells contained 20 µg/ml or 10 µg/ml all of which were determined through pilot data to preserve cell viability. Cells were treated for various durations with final concentration of ethanol 0.1% for all treatments which did not alter cell viability (Supplemental Figure 1).

Metabolic Assay- Cells were seeded overnight in 24-well culture plate from SeaHorse Bioscience (Billerica, MA) at density 5 x 10^5 cells/well (RD cells) or 3 x 10^5 cells/well (C2C12 cells), treated and incubated for 3 or 6 hours as described above. Following treatment, culture media was removed and replaced with XF Assay Media from SeaHorse Bioscience (Billerica, MA) containing 4500mg/L glucose free of CO_2 and incubated at 37°C. Per manufactures' protocol, SeaHorse injection ports were loaded with oligomycin, an inhibitor of ATP synthase which induces maximal glycolytic metabolism and reveals endogenous proton leak (mitochondrial uncoupling) at a final concentration 1.0 µM. Oligomycin addition was followed by the addition of carbonyl cyanide *p*-[trifluoromethoxy]-phenyl-hydrazone (FCCP), an uncoupler of electron transport that induces peak oxygen consumption (an indirect indicator of peak oxidative metabolism) at final concentration 1.25 µM. Rotenone was then added in 1.0 µM final concentration to reveal non-mitochondrial respiration and end the metabolic reactions [29,

30]. Extracellular acidification, an indirect measure of glycolytic capacity, and oxygen consumption, a measure of oxidative metabolism was measured using the SeaHorse XF24 Extracellular Analyzer from SeaHorse Bioscience (Billerica, MA). SeaHorse XF24 Extracellular Analyzer was run using 8 minute cyclic protocol commands (mix for 3 minutes, let stand 2 minutes, and measure for 3 minutes) in triplicate.

Cellular ATP Content- Cells were seeded overnight in a 6-well plate at density 1 x 10^6 cells/well and treated as described above for 24 hours. The cells were lysed in 1% CHAPS lysis buffer from Chemicon (Billerica, MA) in PBS with Ca^{2+} and MG^{2+} and the ATP-containing supernatant was recovered. Samples were allocated into a 96-well plate with a 1:1 dilution of ATP Bioluminescence Reagent from Sigma (St. Louis, MO) with a 100 µM final volume and luminescence was measured and normalized to serial dilutions of ATP. ATP concentrations were normalized to cell density determined through hemocytometey measured by staining cells with trypan blue from Sigma (St Louis, MO) with cell density estimated using a Countess™ cell quantification system from Invitrogen (Carlsbad, CA).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)- Following treatment and incubation as described above, the total RNA was extracted using RNeasy Kit from Qiagen (Valencia, CA), per manufacturer's protocol. Total RNA was quantified by Nanodrop spectrophotometry. cDNA was synthesized from 5000 ng total RNA using the Retroscript™ RT kit from Ambion (Austin, TX) according to manufacturer's instructions. PCR primers were designed using Primer Express software from Invitrogen (Carlsbad, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). Amplification of PGC-1α, nuclear respiratory factor (NRF-1), mitochondrial transcription factor A (TFAM), and glucose transporter 4 (GLUT4) were normalized to the housekeeping gene, TATA Binding Protein (TBP). Table 2

summarizes the forward and reverse primers of each gene. qRT-PCR reactions were performed in triplicate using the LightCycler 480 real-time PCR system from Roche Applied Science, (Indianapolis, IN). SYBR Green based PCR was performed in triplicate using 5000 ng of cDNA per sample; final primer concentrations were 10 µM in a total volume of 30 µl. The following cycling parameters were used: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds, and 60˚C for one minute. Relative expression levels were determined by the ΔΔCp method and compared to the lowest expressing group as previously described [31].

Table 5 Summary of qRT-PCR primers from Integrated DNA Technologies (Coralville, IA). Abbreviations: peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (TFAM), and glucose transporter 4 (GLUT4) and TATA binding protein (TBP). H indicates for primers for human RNA and M indicates primers for mouse RNA.

Primer Name	Forward Sequence	Reverse Sequence
TBP_H	5'-CACGAACCACGGCACTGATT-3'	5'-TTTTCTTGCTGCCAGTCTGGAC-3'
$PGC\text{-}1\alpha_H$	5'-ACCAAACCCACAGAGAACAG-3'	5'-GGGTCAGAGGAAGAGATAAAGTTG-3'
$NRF\text{-}1_H$	5'-GTATCTCACCCTCCAAACCTAAC-3'	5'-CCAGGATCATGCTCTTGTACTT-3'
$TFAM_H$	5'-GGGAAGGAGGGTTGTGTATTT-3'	5'-AGGAGTTAGCCAAACGCAATA-3'
$GLUT4_H$	5'-AAGAATCCCTGCAGCCTGGTAGAA-3'	5'-CCACGGCCAAACCACAACACATAA-3'
TBP_M	5'-GGGATTCAGGAAGACCACATA-3'	5'-CCTCACCAACTGTACCATCAG-3'
$PGC\text{-}1\alpha_M$	5'-GACAATCCCGAAGACACTACAG-3'	5'-AGAGAGGAGAGAGAGAGAGAGA
$NRF\text{-}1_M$	5'-ACCCTCAGTCTCACGACTAT-3'	5'-GAACACTCCTCAGACCCTTAAC-3'
$TFAM_M$	5'-GAAGGGAATGGGAAAGGTAGAG-3'	5'-ACAGGACATGGAAAGCAGATTA-3'
$GLUT4_M$	5'-GGAGGGAGCCTTTGGTATTT-3'	5'-CAGGCGAGGACACTCATCTT-3'

Flow Cytometry- Cells were plated in 6-well plates at a density of 1.0 x 10^6 cells/well treated in triplicate and incubated as previously described above for 24 hours. Following treatment, the media was removed and the cells were re-suspended in pre-warmed media with 200 nM Mitotracker Green from Life Technologies (Carlsbad, CA) and incubated for 45 minutes in a humidified 5% CO_2 atmosphere at 37°C. The cells were pelleted, the media with Mitotracker was removed and the cells were suspended in pre-warmed media. Group mean fluorescence was measured using Facscalibur filtering 488nm. To quantify PGC-1α expression, we used immuonofluorescent staining measured by flow cytometry. Cells were permeabilized with PBS with 0.1% Tween from Sigma (St. Louis, MO) for 10 minutes and blocked for 1 hour with PBS with 0.1% Tween and 3.0% BSA from Sigma (St. Louis, MO). Cells were stained with either an anti-PGC-1α primary polyclonal antibody from Santa Cruz Biotechnologies (Santa Cruz, CA) at 1:200 dilution in PBS with 0.1% BSA overnight. The cells were rinsed with PBS with 0.1% Triton 100X and 3.0% BSA, and secondary anti-rabbit AlexFluor 488 antibody from Invitrogen (Carlsbad, CA) was applied in 1:200 dilutions. Cells were rinsed thoroughly and fluorescence was measured via flow cytometry.

Microscopy and Immunofluorescence- Chamber-slides from BD Bioscience (Sparks, MD), were seeded with 5000 cells/well. To verify PGC-1α protein expression, cells were cultured and treated for 24 hours as described above. Cells were fixed using 3.7% formaldehyde in media, permeabilized with PBS with 0.1% Triton 100X from Sigma (St. Louis, MO) for 10 minutes and blocked for 1 hour with PBS with 0.1% Triton 100X and 3.0% BSA from Sigma (St. Louis, MO). Cells were stained with an anti-PGC-1α primary polyclonal antibody from Santa Cruz Biotechnologies (Santa Cruz, CA) at 1:200 dilution in PBS with 0.1% BSA overnight. The cells were rinsed with PBS with 0.1% Triton 100X and 3.0% BSA, and secondary anti-rabbit

AlexFluor 633 antibody from Invitrogen (Carlsbad, CA) was applied in 1:200 dilution. Slides were mounted with Prolong Gold with DAPI from Invitrogen (Carlsbad, CA) and cured overnight. Cells were imaged using the Axiovert 25 microscope with AxioCam MRc from Zeiss (Thornwood, NY). To verify increased mitochondrial content, the cells were then stained with Mitotracker 200 nM from Invitrogen (Carlsbad, CA) for 45 minutes, and fixed in 3.7% formaldehyde in pre-warmed media. Cells were mounted, cured and imaged as described above.

Cell Viability- Cells were seeded in 96-well plates at density 5,000 cells/well and grown over night. Cells were treated and incubated as previously described for 24 hours. Media and treatment were removed and media containing 10% WST1 was added to each well and were incubated as previously described. Fluorescence was measured 1 hour following WST1 addition using Wallac Victor3V 1420 Multilabel Counter from PerkinElmer (Waltham, MA).

Human Subjects- Healthy male volunteers aged 18-40 years old were eligible for the study. Participants were excluded if they were caffeine naïve, clinically obese (BMI > 30 kg/m^2), if they had known cardiovascular disease, hypertension, or refused to adhere to subject-study procedures. Participants were kindly asked to abstain from caffeine and/or dietary supplement consumption in addition to rigorous exercise at least 48 hours prior to each measurement. Participants were also asked not to smoke at least 12 hours prior to each measure. Subject food intake was recorded the day prior to initial metabolic measurements, and subjects were asked to consume approximately the same meal composition 24 hours prior to the second measurement. Subjects completed a health history questionnaire, food and beverage recall, and informed consent IRB #13-066. Participants reported the usual number of caffeinated beverages (coffee, tea, soft drink, energy drink, etc.) with corresponding serving size, and were asked to list other regularly consumed stimulants (such as those found in dietary supplements or over the counter

medications). Subject height, weight, and resting blood pressure were recorded prior to initial metabolic measurement. Body composition was estimated by 3 site skin-fold measurements (chest, abdominal, and thigh) and estimated body density was used to calculate body fat percent using the Siri equation. Descriptive subject data are listed in Table 3.

Table 6 Summary of subject anthropometric variables and estimated caffeine consumption. † Body fat percent was estimated using 3 site skin fold as described in methods estimated using ExRx body fat calculator. ‡ Caffeine intake was estimated through 24 hour recall food questionnaire and caffeine content of food and beverage list published by the Mayo Clinic available at: http://www.mayoclinic.com/print/caffeine/AN01211/METHOD=print

Subject Variable	Average (±SD)
Age (years)	26.7 (±3.86)
Weight (kg)	77.75 (±8.56)
Height (cm)	175 (±5.0)
BMI (kg/m^2)	25.34 (±2.65)
Body Fat (%) †	14.78 (±6.7)
Lean Body Mass (kg)	65.97 (±6.05)
Caffeine Consumption (mg/day)‡	292.45 (±150.76)

Human Metabolic Measurements- Each subject was asked to participate in 2 double-blinded trials (one placebo filled with dextrose and one RF-treated) resting energy expenditure with at least 48 hours between the two measurements. Treatments were provided in a double-blind fashion by a third party. Following anthropometric measurements, the subject was asked to consume a blinded treatment which was followed by a resting blood pressure measurement. Metabolic measurements were taken at baseline, and both one and two hours following treatment ingestion with blood pressure taken prior to each metabolic measurement. Resting energy expenditure (REE) and respiratory exchange ratio (RER) were measured using a TrueOne 2400 metabolic measurement system from ParvoMedics (Sandy, UT) following overnight fast similar to previous reports [16]. The ParvoMedics metabolic cart was calibrated daily per manufacturer guidelines prior to each trial. Participants rested in a supine position in a comfortable ambient temperature in a quiet and dark room. Expired gas was collected by placing a clear hood over the participant's head and upper torso area with plastic seal secured under the subject. Data were collected for approximately 30 minutes per measurement and the 5 minute duration which produced the lowest variability of REE data was used for values of REE and RER for all tests. REE data was transformed into kcals/kg/day for both total body weight and lean body mass. Flow rate was monitored by a designated research assistant during the course of the test and maintained at a rate of 1–1.2% expired carbon dioxide per manufacture's protocol.

Statistical Analysis- Cellular metabolic assays, metabolic gene expression, flow cytometry, and cell viability were analyzed using ANOVA with Dunnett's post hoc test and pairwise comparisons were used to compare treatments with control. Microscopy was analyzed using student's t test. Human REE (kcals/kg/day), RER (VCO_2/VO_2), and both systolic and diastolic

blood pressure response were analyzed using paired t-tests for each time point and trial averages. Correlations between age, BMI, and self-reported caffeine consumptions were analyzed using linear regression. Values of $p < 0.05$ indicated statistical significance in all tests and Prism from GraphPad (La Jolla, CA) was used to perform all statistical analyses.

Results

RF Enhances Oxidative Metabolism In Vitro- To evaluate the effects of RF treatment on oxidative metabolism in cultured myocytes, we measured oxygen consumption. Treatment with RF for either 3 or 6 hours significantly increased basal oxygen consumption in both cell models (Figure 1A). Peak oxidative metabolism was also significantly increased in both cell models following 3 hours of treatment, but was only significantly elevated in the C2C12 cell model following 6 hours of treatment suggesting a differing response between the models with time-dependence (Figure 1B). In correspondence with basal oxidative metabolism, cells treated with RF also exhibited significantly greater mitochondrial uncoupling (proton leak) (Figure 1C).

RF Alters Glycolytic Metabolism and Metabolic Reliance In Vitro- To investigate the effects of RF treatment on glycolytic metabolism, we measured extracellular acidification rate. Interestingly, RF-treated RD cells either displayed no change or a reduced glycolytic rate while RF-treated C2C12 cells exhibited a significant increase in basal glycolysis compared with corresponding controls (Figure 1D). This discrepancy between the two cell models was also seen during chemically induced peak glycolysis. Specifically, RD cells treated with RF exhibited significantly reduced glycolysis at both 3 and 6 hours. Conversely, C2C12 cells treated with RF showed significantly elevated peak glycolytic metabolism (Figure 1E). Because cells were culture in high-glucose media (per ATCC recommendations), and because RD cells exhibited a

dynamic increase in oxidative metabolism, we interpret the reduction in cellular acidification to indicate more complete carbohydrate metabolism which we have previously described [24]. Despite discrepancy in glycolytic response between the 2 cell models, both cell types exhibited a significantly increased reliance on oxidative metabolism (expressed as a ratio of OCR:ECAR) when compared with respective controls (Figure 1F).

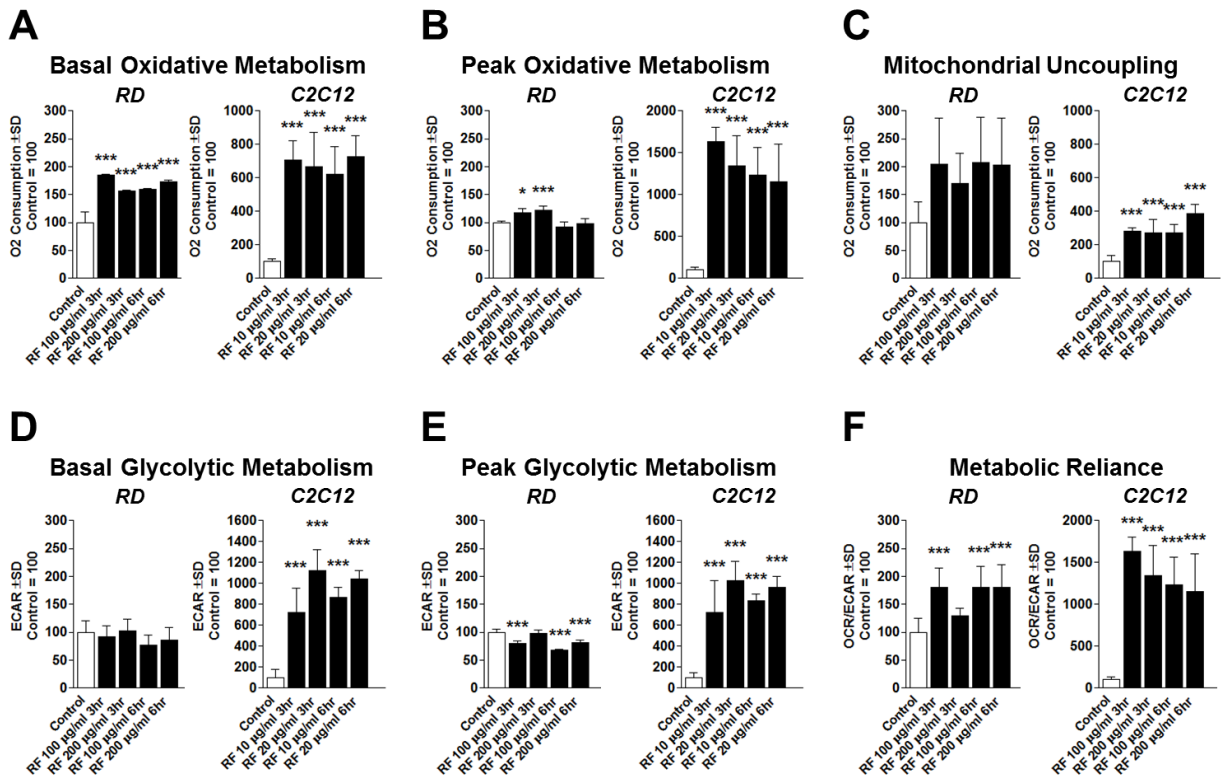

Figure 4 Oxidative Metabolism (A) Basal oxidative metabolism indicated by oxygen consumption rate (OCR) of RD (left) cells treated with either ethanol control or RF at 100 µg/ml or 200 µg/ml for 3 or 6 hours and C2C12 (right) cells treated with either ethanol control or RF at 10 µg/ml or 20 µg/ml for 3 or 6 hours. (B) Peak OCR of RD and C2C12 cells following addition of oligomycin following treatment as described in A. (C) Mitochondrial uncoupling (endogenous mitochondrial proton leak) of RD and C2C12 cells treated as described in A. (D) Basal glycolytic metabolism indicated by extracellular acidification rate (ECAR) of RD (left) cells treated as described above. (E) Peak ECAR of RD and C2C12 cells following the addition of oligomycin following treatment as described above. (F) Metabolic reliance expressed as a ratio of OCR:ECAR.

NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and * indicates $p < 0.001$ compared with control.**

RF Induces Metabolic Gene Expression In Vitro- To investigate the effects of RF treatment on metabolic gene expression, we measured relative RNA expression of PGC-1α, NRF-1, TFAM, and GLUT4 expression normalized to TBP following treatment with RF for 4, 12, and 24 hours. RD cells treated with RF exhibited significantly elevated PGC-1α expression at initial time points (Figure 2A) while C2C12 cells only exhibited elevated PGC-1α expression following 4 hours of treatment (Figure 2B). NRF-1 and TFAM, downstream targets of PGC-1α were also significantly induced by RF after treatment of both cell models. Additionally, GLUT4 expression was also elevated above control levels at various time points for both cell models the most notable of which occurred at 24 hours.

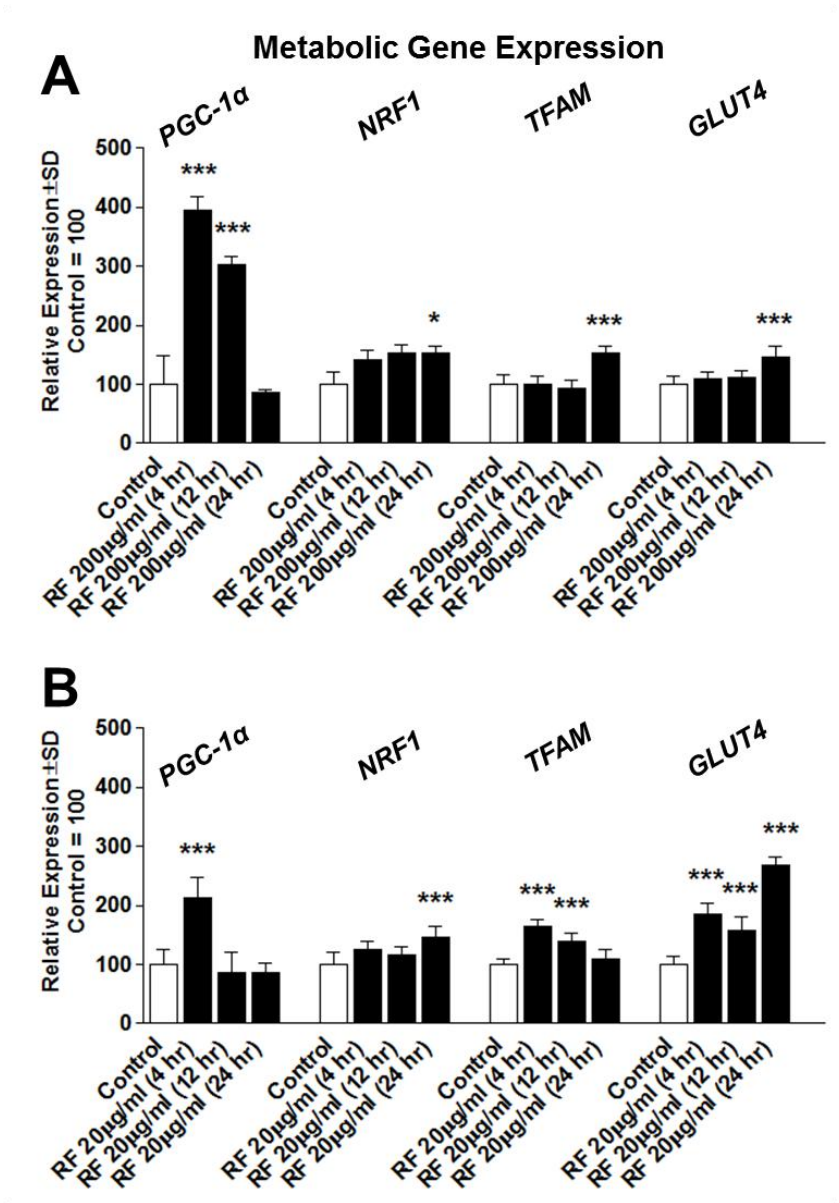

Figure 5 Metabolic Gene Expression. (A) Gene expression of RD cells treated with either ethanol control or RF at 100 µg/ml or 200 µg/ml for 4, 12, or 24 hours of PGC-1*α*, NRF-1, TFAM, and GLUT4. (B) Gene expression of C2C12 cells treated with either ethanol control or RF at 10 µg/ml or 20 µg/ml for 4, 12, or 24 hours of PGC-1*α*, NRF-1, TFAM, and GLUT4.

NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and * indicates $p < 0.001$ compared with control.**

RF Induces Mitochondrial Biosynthesis Through PGC-1α- In order to assess changes in metabolic gene expression, we quantified PGC-1α expression by flow cytometry. RF treatment of RD cells significantly induced PGC-1α protein expression in a dose-dependent manner compared with the control (Figure 3A). C2C12 cells treated with RF also displayed amplified PGC-1α expression (Figure 3B). To verify our flow cytometry observations, we measured PGC-1α protein expression by immunofluorescent confocal microscopy (Figure 3C and D, respectively). Additionally, heightened PGC-1α expression in RF treated cells was also associated with significantly elevated mitochondrial content in both cell models (Figure 4A and B). In order to verify flow cytometry observations in both RD and C2C12 cells, we measured mitochondrial content by immunofluorescent via confocal microscopy (Figure 4C and D, respectively). In addition, RF treatment caused a significant reduction in cellular ATP content for C2C12 myocytes without altering ATP content of RD cells (Figure 4E).

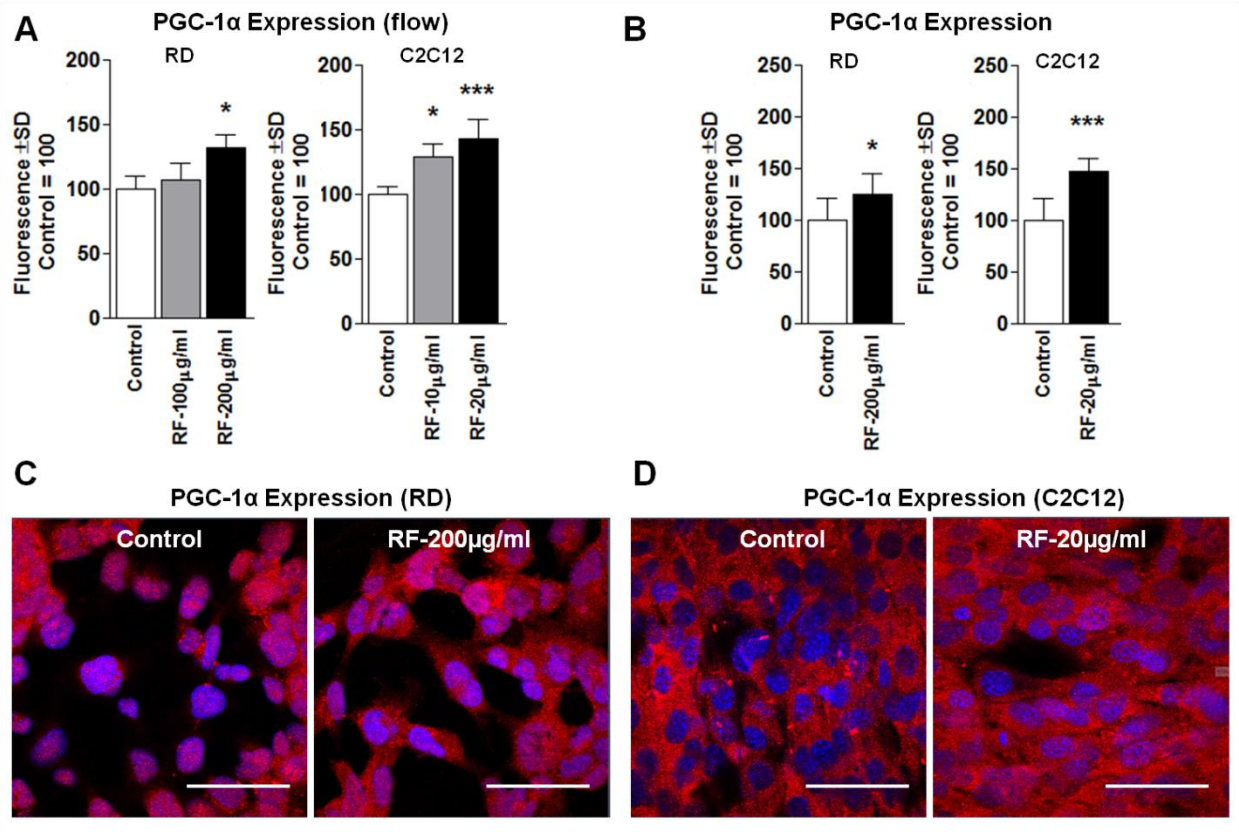

Figure 6 PGC-1α Protein Expression (A) Group mean log fluorescence from flow cytometry of RD (left) and C2C12 cells (right) treated as described in methods for 24 hours stained with PGC-1α primary antibody and AlexaFluor 488 secondary antibody. (B) Group mean log fluorescence of confocal microscopy of RD cells treated as previously described for 24 hours. (C and D) Representative images from fluorescent confocal microscopy of RD cells (C) and C2C12 cells (D) treated as described earlier. Fluorescent measurements were performed using *N* = 7 cells/treatment which were stained with PGC-1α primary antibody and AlexaFluor 533 secondary antibody (red) and DAPI (blue).

NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and * indicates $p < 0.001$ compared with control. White measurement bar represents 50 µm.**

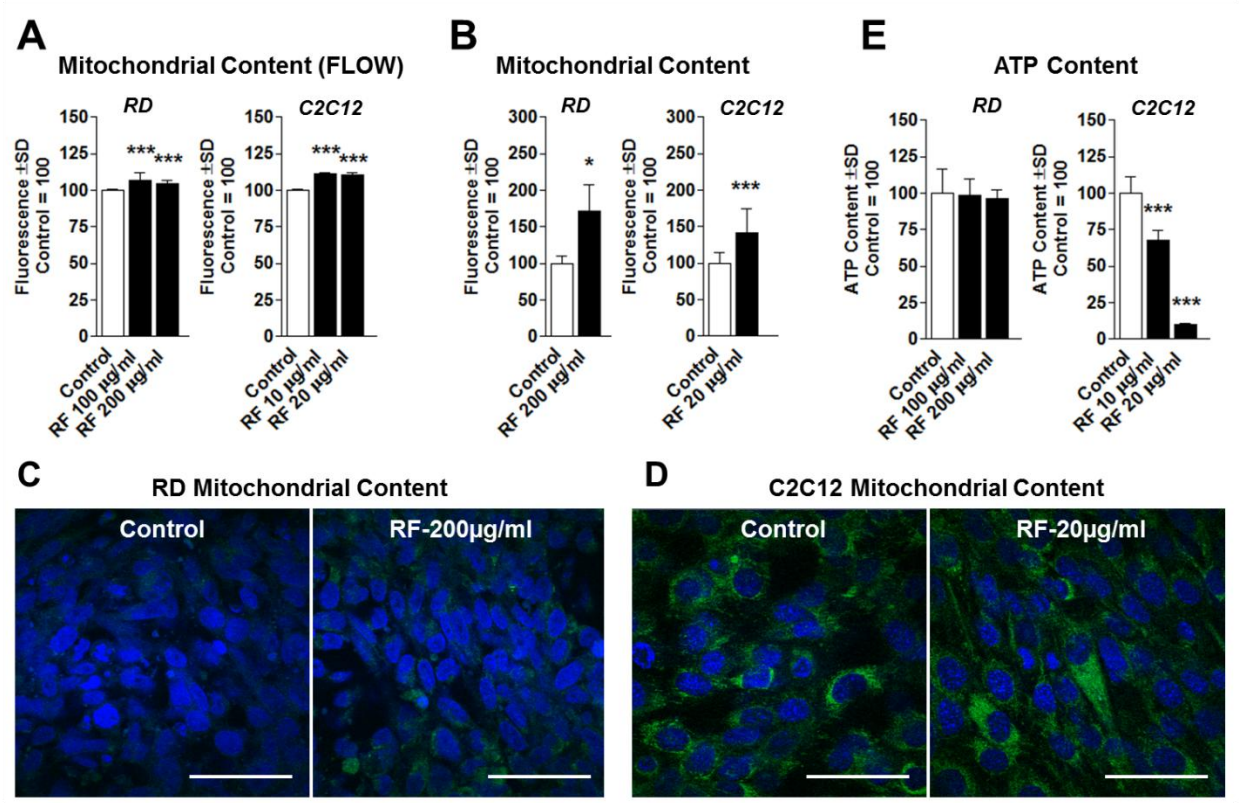

Figure 7 Mitochondrial Content (A) Group mean log fluorescence from flow cytometry of RD (left) and C2C12 cells (right) treated as described in methods for 24 hours stained with mitotacker green. (B) Group mean log fluorescence of confocal microscopy of RD cells treated as previously described for 24 hours. (C and D) Representative images from fluorescent confocal microscopy of RD cells (C) and C2C12 cells (D) treated as described above. Fluorescent measurements were performed using *N* = 7 cells/treatment which were stained with mitotracker (green) and DAPI (blue). (E) Cellular ATP content following treatment of either RD cells (left) or C2C12 cells (right) with RF at various doses for 24 hours.

NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and * indicates $p < 0.001$ compared with control. White measurement bar represents 50 µm.**

RF Enhances Resting Energy Expenditure In Vivo- To evaluate the effects of RF consumption on metabolic rate in humans, we measured REE following ingestion of either placebo or a single serving of RF (in a double-blinded fashion). RF increased REE during initial measurement, and 1 and 2 hours post ingestion as well as average energy expenditure for the entire trial compared with the control treatment (Figure 5A). RF consumption was associated with an average increase in daily REE of 159.7 ±89.7 kcal/day. Unexpectedly, RER remained unchanged throughout the trial between RF and placebo treatment (Figure 5B). To investigate the contribution of subject variables (summarized in Table 1) on response to RF, we assessed relationships between age, BMI, and prior self-reported caffeine consumption, none of which were significantly correlated with RF response (Figure 6A, B, and C, respectively). Additionally, we measured the effects of RF consumption on resting blood pressure. RF treatment elevated systolic blood pressure 2 hours following ingestion, as well as the average systolic blood of subjects throughout the trial (Figure 6D). Diastolic blood pressure remained unchanged for each time point and for the average of the trial (Figure 6E).

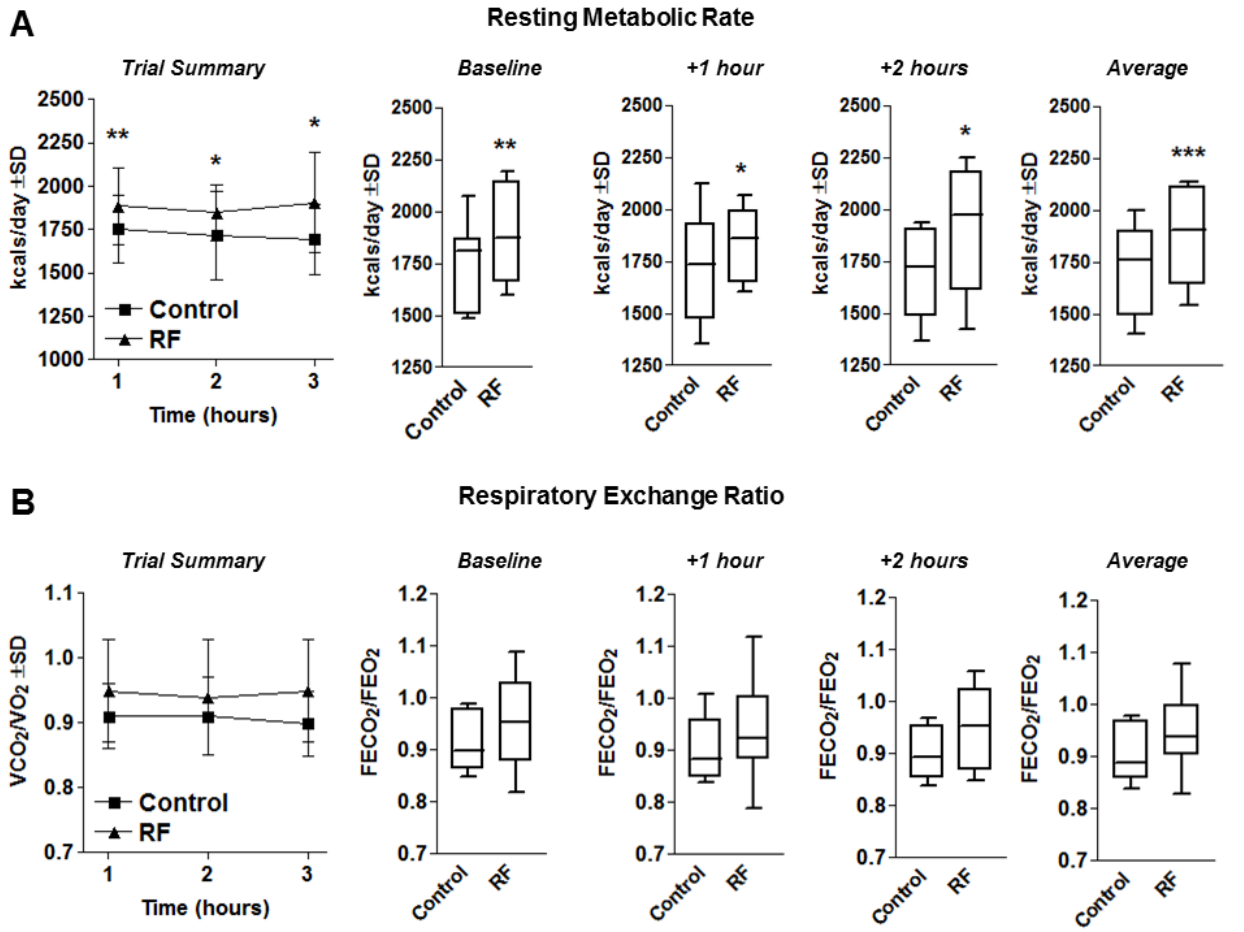

Figure 8 Metabolic Rate and Substrate Utilization (A) Resting energy expenditure (REE) of human male subjects following ingestion of either a placebo (control) or a single serving of RF. (B) Respiratory exchange ratio (RER) of human male subjects following ingestion of either a placebo (control) or a single serving of RF. NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and * indicates $p < 0.001$ compared with control.**

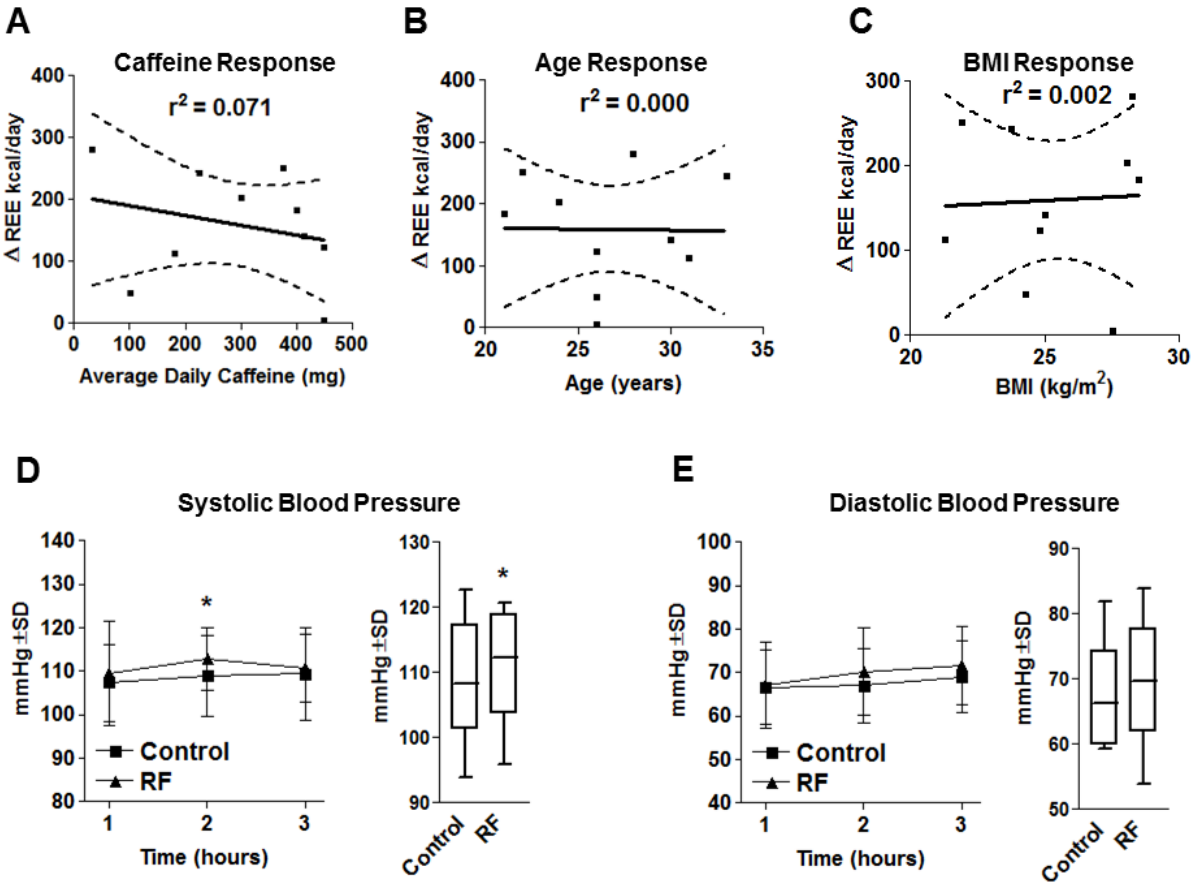

Figure 9 Correlation of Subject Variables and Metabolic Response (A-C) Change in resting energy expenditure (REE) of human male subjects following ingestion of either a placebo (control) or a single serving of RF correlated with caffeine consumption, age, or BMI, respectively. (D) Systolic blood pressure of human male subjects following ingestion of either a placebo (control) or a single serving of RF. (E) Diastolic blood pressure of human male subjects following ingestion of either a placebo (control) or a single serving of RF. NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and * indicates $p < 0.001$ compared with control.**

Discussion

Previously, published data was scarce regarding the effects of RF on metabolism. Our data are among the first observations to describe both the molecular and physiological effects of RF which include increased cellular metabolism with enhanced mitochondrial uncoupling *in vitro*. Interestingly, our observations demonstrate that RF treated muscle cells consistently exhibited significantly greater basal oxidative accompanied by a significant increase in peak oxidative capacity. Our data also demonstrates that 24 hour treatment with RF significantly induced PGC-1α protein which was also accompanied by a significant increase in mitochondrial content in both cell models. An interesting finding is that RD cells tolerated ten times higher treatments than the non-malignant myoblasts, suggesting that RD cells are substantially more resilient. This alteration in glycolytic metabolism may also be a result of the cancerous phenotype that RD cells possess, which selectively prefer glycolytic metabolism. Additionally, in many cases the lower dose tested in both cell models elicited an enhanced response over the highest dose tested suggesting an optimal and saturable effect *in vitro*. Cumulatively, the *in vitro* findings are similar to several other studies performed by our lab which showed that various dietary constituents can increase cell metabolism and mitochondrial content in skeletal muscle cells [8, 22, 23]. These observations also parallel those of other previous investigations that measured the effects of dietary stimulants such as caffeine on similar metabolic parameters in L6 myotubes and C2C12 myocytes [19, 32, 33].

In agreement with previous *in vitro* observations using similar dietary supplements, RF increases metabolic rate in healthy adult male subjects [16-18]. Several similarities exist between supplements designed to stimulate metabolism including stimulants such as caffeine which have previously been documented to increase metabolism in humans [4, 5, 7, 9, 34]. We have recently

discussed many of these similarities including ingredient composition and effect on resting energy expenditure [35]. Despite these findings, it is unclear to what extent energy expenditure is increased for the purpose of weight loss. Because our measurements were limited to 3 hours, several questions remain unanswered about the effects of RF. First, at what point following a single dose does resting energy expenditure return to normal? Are these effects diminished with ongoing use (i.e. sensitization)? Are these effects also seen in older adults and women? Will increases in systolic blood pressure resolve with use? Our data adds to the current literature which supports the hypothesis that dietary composition may play a vital role in human energy homeostasis and energy balance. Proprietary blends consisting of multiple plant extracts potentially offer numerous diverse phytochemicals which may be useful in altering energy expenditure, although promotional advertising makes much of current information difficult for consumers to trust. Currently there is an immense interest in both private and commercial sectors for exploring extracts (such as green tea) and food-stuff for the benefit of metabolic disease including obesity and diabetes supporting the need for ongoing research in dietary supplements and functional food chemicals [34].

Conclusion

Dietary supplements are generally regarded as ineffective, under-researched, and potentially risky by the medical community. Despite this general consensus, there is an increasing interest in alternative methods to increase metabolism and fat loss. Our observations support the hypothesis that RF may be effective at increasing metabolic rate *in vitro* and *in vivo*. Although many supplements are commercially available for consumption and are advertised to increase metabolism and cause weight loss, few supplements have non-biased data supporting claims of

efficacy. There are many therapies that are currently recommended to promote weight loss. However, dietary supplements are generally not regarded as a part of the standard of care for weight loss. Further research is needed to elucidate the full effects of habitual RF consumption on human metabolism and health.

Competing Interests

All authors and contributors declare no conflict of interests.

Acknowledgements

Funding was provided by the University of New Mexico Summer 2012 Office of Graduate Studies Research, Project and Travel Grant, and through Department of Biochemistry and Molecular Biology Faculty Research Allocation Funds provided by Kristina Trujillo Ph.D. We would like to thank the University of New Mexico Department of Biochemistry and Molecular Biology for their assistance in this work.

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Chapter 4

Implications for Dietary Components in Metabolic Disease

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Current research has outlined many of the potential benefits that dietary ingredients may provide for metabolic disease. Stimulation of metabolic gene expression (including PGC-1α expression) via dietary metabolites may result in increase in oxidative capacity, which may provide some benefit for metabolic disease including diabetes and obesity. Further investigation is required to elucidate the full potential efficacy of dietary PGC-1 stimulators for metabolic disease. Dietary supplements are generally regarded as ineffective, under-researched, and potentially risky by the medical community. Despite this general consensus, there is an increasing interest in alternative methods to increase metabolism and fat loss. Our observations, along with those generated from several other groups support the hypothesis that dietary supplements may effectively increase metabolic rate. Many supplements are commercially available for consumption and are advertised to increase metabolism and cause weight loss. Few supplements have non-biased data supporting claims of efficacy. From the available evidence, it appears that commercially available dietary supplements advertised to stimulate metabolism have the propensity to increase metabolic rate, at least immediately following ingestion. Despite significant increases in resting energy expenditure, it is doubtful that commercially available thermogenic products stimulate metabolism more than consumption of food products containing equivocal content of caffeine/stimulants and/or polyphenols. Moreover, it should be mentioned that increases in metabolism induced by food or dietary supplements are small, contributing only subtly to metabolic rate. Further research is needed to elucidate the full effects of habitual dietary supplement consumption on human metabolism and health. Specifically, further research to elucidate the effects that dietary stimulators of metabolism have on various demographics including female and obese subjects. This includes evidence which speaks to ongoing safety and therapeutic doses for both men and women of various ages and body sizes. Lastly, future

investigation is required to identify other molecular effects that dietary supplements have, which could lead to identification of future therapeutic targets.

Appendix 1 Individual subject variables

Subject Variables								
Subject	**Weight (kg)**	**Height (m)**	**BMI**	**Fat%**	**LBM(kg)**	**Caffeine (mg)**	**Δ REE**	**age**
1.00	89.80	1.78	28.25	13.90	77.32	32.00	281.35	28.00
2.00	68.60	1.77	21.90	10.63	61.31	375.00	251.05	22.00
3.00	71.60	1.84	21.26	5.50	67.66	180.00	113.01	31.00
4.00	69.80	1.67	25.03	8.19	64.08	412.50	141.86	30.00
5.00	81.40	1.72	27.51	20.00	65.12	450.00	6.24	26.00
6.00	75.60	1.75	24.83	6.68	70.55	450.00	123.14	26.00
7.00	71.00	1.71	24.28	16.40	59.36	100.00	49.15	26.00
8.00	86.00	1.75	28.08	24.00	65.36	300.00	203.41	24.00
9.00	72.80	1.75	23.77	21.25	57.33	225.00	244.14	33.00
10.00	90.91	1.79	28.50	21.25	71.59	400.00	183.61	21.00
Ave	**77.75**	**1.75**	**25.34**	**14.78**	**65.97**	**292.45**	**159.70**	**26.70**
SD	**8.56**	**0.05**	**2.65**	**6.76**	**6.05**	**150.76**	**89.67**	**3.86**

Appendix 2 Change in individual subject resting energy expenditure

REE (kcal/day)										
	Baseline		1 hr		2 hr		Average		STDEV	
Subject	**Control**	**RF**	**Control**	**RF**	**Control**	**RF**	**Control**	**RF**	**Control**	**RF**
1.00	1853.95	2165.73	1830.14	2001.86	1871.85	2232.41	1851.98	2133.33	20.92	118.64
2.00	1830.38	1861.50	1542.10	1845.61	1586.66	2005.18	1653.04	1904.10	155.18	87.90
3.00	1770.78	1793.33	1637.72	1867.95	1703.10	1789.36	1703.87	1816.88	66.53	44.27
4.00	1483.18	1602.60	1357.21	1604.52	1365.64	1424.49	1402.01	1543.87	70.42	103.39
5.00	1884.07	1924.05	1873.63	1854.27	1937.48	1935.59	1898.39	1904.64	34.25	44.00
6.00	2071.44	2191.18	1980.51	2069.46	1935.22	2095.95	1995.72	2118.86	69.37	64.02
7.00	1511.82	1600.64	1561.92	1608.00	1491.10	1503.66	1521.62	1570.77	36.41	58.23
8.00	1843.55	2122.64	2121.38	1947.07	1745.37	2250.84	1903.43	2106.85	195.03	152.50
9.00	1498.28	1714.37	1408.60	1691.70	1479.76	1713.01	1462.22	1706.36	47.34	12.71
10.00	1787.48	1879.74	1845.31	1985.00	1803.29	2122.18	1812.03	1995.64	29.89	121.57
Ave	**1753.49**	**1885.58**	**1715.85**	**1847.54**	**1691.95**	**1907.27**	**1720.43**	**1880.13**	**72.54**	**80.72**
SD	**194.45**	**218.39**	**252.15**	**164.33**	**202.79**	**291.27**	**205.22**	**218.58**	**57.52**	**43.35**

Appendix 3 Change in individual subject respiratory exchange ratio

RER (VCO_2/VO_2)										
	Baseline		1 hr		2 hr		Average		STDEV	
Subject	Control	RF	Control	RF	Control	RF	Control	RF	Control	RF
1.00	0.90	0.85	0.88	0.85	0.89	1.06	0.89	0.92	0.01	0.12
2.00	0.90	0.92	0.84	0.92	0.86	0.97	0.87	0.94	0.03	0.03
3.00	0.93	1.09	0.95	1.12	0.90	1.04	0.93	1.08	0.03	0.04
4.00	0.97	0.94	0.96	0.92	0.97	0.86	0.97	0.91	0.01	0.04
5.00	0.85	0.97	0.84	0.92	0.84	0.94	0.84	0.94	0.01	0.03
6.00	0.86	0.82	0.86	0.79	0.85	0.88	0.85	0.83	0.01	0.04
7.00	0.87	1.01	0.89	1.05	0.89	0.91	0.89	0.99	0.01	0.07
8.00	0.99	1.05	1.01	0.96	0.95	1.01	0.98	1.01	0.03	0.05
9.00	0.89	0.91	0.86	0.93	0.91	0.85	0.88	0.90	0.03	0.04
10.00	0.99	0.97	0.96	0.96	0.96	0.99	0.97	0.97	0.02	0.01
Ave	**0.91**	**0.95**	**0.91**	**0.94**	**0.90**	**0.95**	**0.91**	**0.95**	**0.02**	**0.05**
SD	**0.05**	**0.08**	**0.06**	**0.09**	**0.05**	**0.08**	**0.05**	**0.07**	**0.01**	**0.03**

Appendix 4 Change in individual subject Systolic blood pressure

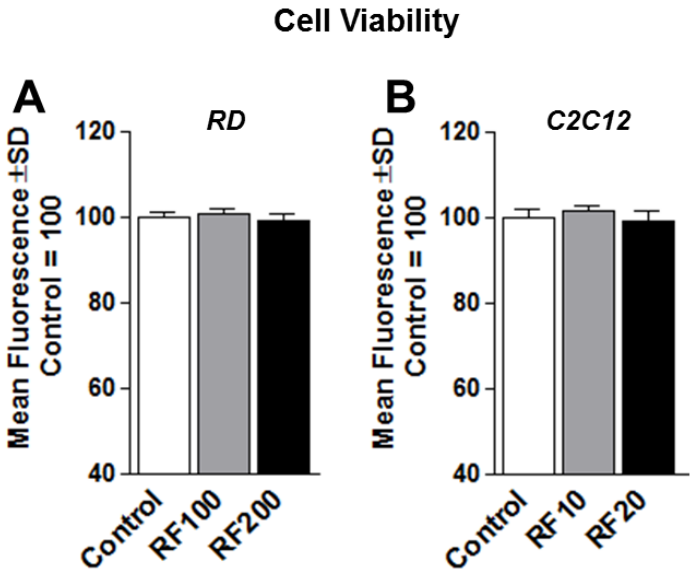
Systolic BP (mmHg)										
	Baseline		1 hr		2 hr		Average		STDEV	
Subject	Control	RF	Control	RF	Control	RF	Control	RF	Control	RF
1.00	88.00	82.00	98.00	104.00	96.00	102.00	94.00	96.00	5.29	12.17
2.00	108.00	114.00	112.00	120.00	106.00	112.00	108.67	115.33	3.06	4.16
3.00	106.00	112.00	112.00	116.00	106.00	118.00	108.00	115.33	3.46	3.06
4.00	106.00	98.00	94.00	102.00	94.00	102.00	98.00	100.67	6.93	2.31
5.00	110.00	108.00	112.00	112.00	110.00	108.00	110.67	109.33	1.15	2.31
6.00	116.00	122.00	124.00	120.00	118.00	120.00	119.33	120.67	4.16	1.15
7.00	120.00	120.00	118.00	118.00	130.00	120.00	122.67	119.33	6.43	1.15
8.00	104.00	116.00	102.00	104.00	108.00	106.00	104.67	108.67	3.06	6.43
9.00	102.00	106.00	104.00	112.00	108.00	102.00	104.67	106.67	3.06	5.03
10.00	114.00	118.00	114.00	120.00	118.00	118.00	115.33	118.67	2.31	1.15
Ave	**107.40**	**109.60**	**109.00**	**112.80**	**109.40**	**110.80**	**108.60**	**111.07**	**3.89**	**3.89**
SD	**8.85**	**12.07**	**9.30**	**7.19**	**10.63**	**7.73**	**8.94**	**8.29**	**1.82**	**3.41**

Appendix 5 Change in individual subject diastolic blood pressure

Diastolic BP (mmHg)										
	Baseline		1 hr		2 hr		Average		STDEV	
Subject	Control	RF	Control	RF	Control	RF	Control	RF	Control	RF
1.00	56.00	56.00	58.00	62.00	64.00	62.00	59.33	60.00	4.16	3.46
2.00	64.00	66.00	68.00	82.00	68.00	76.00	66.67	74.67	2.31	8.08
3.00	70.00	62.00	62.00	64.00	66.00	70.00	66.00	65.33	4.00	4.16
4.00	56.00	54.00	62.00	52.00	64.00	56.00	60.67	54.00	4.16	2.00
5.00	60.00	68.00	62.00	70.00	56.00	76.00	59.33	71.33	3.06	4.16
6.00	66.00	58.00	74.00	64.00	70.00	70.00	70.00	64.00	4.00	6.00
7.00	82.00	84.00	80.00	84.00	84.00	84.00	82.00	84.00	2.00	0.00
8.00	66.00	70.00	68.00	70.00	64.00	64.00	66.00	68.00	2.00	3.46
9.00	68.00	76.00	56.00	74.00	76.00	76.00	66.67	75.33	10.07	1.15
10.00	78.00	78.00	80.00	80.00	78.00	82.00	78.67	80.00	1.15	2.00
Ave	**66.60**	**67.20**	**67.00**	**70.20**	**69.00**	**71.60**	**67.53**	**69.67**	**3.69**	**3.45**
SD	**8.54**	**9.99**	**8.60**	**10.09**	**8.23**	**8.93**	**7.65**	**9.22**	**2.49**	**2.37**

Appendix 6 Individual subject treatment schedule (determined randomly by a third party via random.org)

Subject #	Trial 1	Trial 2
1	Placebo	RF
2	RF	Placebo
3	Placebo	RF
4	RF	Placebo
5	Placebo	RF
6	RF	Placebo
7	RF	Placebo
8	Placebo	RF
9	Placebo	RF
10	RF	Placebo



Supplemental Figure 1. (A) Cell viability of RD cells treated with RF at either 100 µM or 200 µM for 24 hours. (B) Cell viability of C2C12 cells treated with RF at either 10 µM or 20 µM for 24 hours.