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Original article

Effect of novel dietary supplement on metabolism *in vitro* and *in vivo*

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ABSTRACT

Obesity is an increasingly prevalent and preventable morbidity with multiple behavioral, surgical and pharmacological interventions currently available. Commercial 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. We describe a commercially available dietary supplement (purportedly containing caffeine, catechins, and other metabolic stimulators) on resting metabolic rate in humans, and on metabolism, mitochondrial content, and related gene expression *in vitro*. Human males ingested either a placebo or commercially available supplement (RF) in a randomized double-blind placebo-controlled cross-over fashion. Metabolic rate, respiratory exchange ratio, and blood pressure were measured hourly for 3 h post-ingestion. To investigate molecular effects, human rhabdomyosarcoma cells (RD) and mouse myocytes (C2C12) were treated with various doses of RF for various durations. RF enhanced energy expenditure and systolic blood pressure in human males without altering substrate utilization. In myocytes, RF enhanced metabolism, metabolic gene expression, and mitochondrial content suggesting RF may target common energetic pathways which control mitochondrial biogenesis. RF appears to increase metabolism immediately following ingestion, although it is unclear if RF provides benefits beyond those provided by caffeine alone. Additional research is needed to examine safety and efficacy for human weight loss.

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1. Introduction

Obesity is an increasingly prevalent morbidity with nearly two-thirds of adult Americans overweight, with 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.

Ripped Freak® (RF) from PharmaFreak (Toronto, Canada) is one such commercially available dietary supplement advertised to act as a thermogenic agent, although there appear to be no previously published data on its efficacy. RF is specifically purported 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 that purportedly comprise RF's proprietary blend

Abbreviations: GLUT4, Glucose transporter 4; TFAM, Mitochondrial transcription factor A; NRF-1, Nuclear respiratory factor; PGC-1α, Proliferator-activated receptor γ coactivator-1α; REE, Resting energy expenditure; RER, Respiratory exchange ratio; TBP, TATA Binding Protein.

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include caffeine, green tea (綠茶lǜ chá), raspberry ketones, and capsaicin; many of which have been previously linked to increased metabolic rate (Table 1).

Previously, caffeine has been shown 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 may 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 have 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 is 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] Because skeletal muscle contributes largely to total energy expenditure, and because it is highly responsive to a variety of stimuli, skeletal muscle represents a meaningful target for metabolic disease such as obesity and diabetes.[28] This work seeks to evaluate the acute effects of RF on energy expenditure in healthy male human subjects, as well as investigate the molecular effects of RF on skeletal muscle *in vitro*.

Table 1
Purported composition of Ripped Freak®.

Ripped Freak® Formula 766.6 mg[a]
Fat burning metabolism amplifier 400 mg[a]
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 200 mg[a]
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 166.6 mg[a]
4-(4-Hydroxyphenyl)-2-Methyl Ethyl Ketone (Raspberry Ketone)
4-(4-Hydroxyphenyl)-2-Butanone Methyl Gallate Ester (Raspberry Ketone – Gallic Acid)

[a] Percent daily value not established.

2. Materials and methods

2.1. In vivo

2.1.1. Human subjects

Healthy male volunteers aged 18–40 years 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. Ten ($n = 10$) eligible and willing participants were asked to abstain from caffeine and/or dietary supplement consumption in addition to rigorous exercise at least 48 h prior to each measurement. Participants were also asked not to consume anything but water and not to smoke at least 12 h prior to each study visit. Subject food intake was recorded the day prior to initial metabolic measurements, and subjects were asked to consume approximately the same meal composition 24 h prior to the second measurement. Subjects completed a health history questionnaire, food and beverage recall, and informed consent as approved by the Institutional Review Board (HRPO #13-066) and HIPAA. Participants reported their usual intake 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 2.

2.1.2. Human metabolic measurements

Each subject was asked to participate in two trials in a double-blind-placebo-controlled cross-over design consisting of two treatments (one placebo filled with dextrose and one serving of RF constituting the actual treatment) for measurement of resting energy expenditure with at least 48 h 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 at both 1 and 2 h following treatment ingestion with blood pressure assessed prior to each metabolic measurement. Resting energy expenditure (REE) and respiratory exchange ratio (RER) were measured using a metabolic measurement system (TrueOne 2400 from Parvo Medics, Sandy, UT) following an overnight fast similar to previous reports.[16] The 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. 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. Data were collected for approximately 30 min per measurement and the 5 min duration which produced the lowest variability of REE data was used for values of REE and RER for all tests. REE data were transformed into kcals/kg/day for both total body weight and lean body mass.

2.2. In vitro

2.2.1. Cell culture

Human rhabdomyosarcoma cells (RD) and mouse myocytes (C2C12) were purchased from ATCC (Manassas, VA). Cells were

Table 2
Summary of subject ($N = 10$) anthropometric variables and estimated caffeine consumption.

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 (%)[a]	14.78 (±6.7)
Lean body mass (kg)	65.97 (±6.05)
Caffeine consumption (mg/day)[b]	292.45 (±150.76)

[a] Body fat percent was estimated using the Siri equation from 3 site skin fold measurements.
[b] Caffeine intake was estimated through 24 h 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.

cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4500 mg/L glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin, in a humidified 5% CO_2 atmosphere at 37 °C. 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. Cells were treated for various durations with a final concentration of ethanol of 0.1% for all treatments, which did not alter cell viability (data not shown).

2.2.2. Metabolic assay

Cells were seeded overnight in 24-well culture plate from SeaHorse Bioscience (Billerica, MA) at density 5×10^5 cells/well (RD cells) or 3×10^5 cells/well (C2C12 cells), treated and incubated for 3 or 6 h as described above. Following treatment, culture media was removed and replaced with XF Assay Media from SeaHorse Bioscience (Billerica, MA) containing 4500 mg/L glucose free of HCO_3^- and incubated at 37 °C. Per manufacturers' 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 min cyclic protocol commands (mix for 3 min, let stand 2 min, and measure for 3 min) in triplicate.

2.2.3. Cellular ATP content

Cells were seeded overnight in a 6-well plate at density 1×10^6 cells/well and treated as described above for 24 h. 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 hemocytometery 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).

2.2.4. 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 3 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 min followed by 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Relative expression levels were determined by the ΔΔCp method and compared to the lowest expressing group as previously described.[31]

2.2.5. Flow cytometry

Cells were plated in 6-well plates at a density of 1.0×10^6 cells/well treated in triplicate and incubated as previously described above for 48 h. 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 min 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 488 nm.

2.2.6. Microscopy and immunofluorescence

To verify increased mitochondrial content, the cells were then stained with MitoTracker 200 nM from Invitrogen (Carlsbad, CA) for 45 min, rinsed thoroughly, and fixed in 3.7% formaldehyde in pre-warmed media. Cells were mounted, cured and imaged as described above. 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).

2.2.7. Cell viability

Cells were seeded in 96-well plates at density 5000 cells/well and grown over night. Cells were treated and incubated as previously described for 24 h. 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 h following WST1 addition using Wallac Victor3V 1420 Multilabel Counter from PerkinElmer (Waltham, MA).

2.2.8. 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 (kcal/hr/kg), RER (VCO_2/VO_2), and both systolic and diastolic blood pressure responses were analyzed by two-way repeated measures ANOVA. Correlations between age, BMI, and self-reported caffeine consumptions were analyzed using

Table 3
Summary of qRT-PCR primers from Integrated DNA Technologies (Coralville, IA).

Primer name	Forward sequence	Reverse sequence
TBP_H	5′-CACGAACCACGGCACTGATT-3′	5′-TTTTCTTGCTGCCAGTCTGGAC-3′
PGC-1α_H	5′-ACCAAACCCACAGAGAACAG-3′	5′-GGGTCAGAGGAAGAGATAAAGTTG-3′
NRF-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-1α_M	5′-GACAATCCCGAAGACACTACAG-3′	5′-AGAGAGGAGAGAGAGAGAGAGA
NRF-1_M	5′-ACCCTCAGTCTCACGACTAT-3′	5′-GAACACTCCTCAGACCCTTAAC-3′
$TFAM_M$	5′-GAAGGGAATGGGAAAGGTAGAG-3′	5′-ACAGGACATGGAAAGCAGATTA-3′
$GLUT4_M$	5′-GGAGGGAGCCTTTGGTATTT-3′	5′-CAGGCGAGGACACTCATCTT-3′

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.

linear regression. Values of $p < 0.05$ indicated statistical significance in all tests. Prism from GraphPad (La Jolla, CA) was used to perform all statistical analyses.

3. Results

3.1. *In vivo*

3.1.1. *RF enhances resting energy expenditure*

To evaluate the effects of RF consumption on metabolic rate in humans, we measured REE following ingestion of either a single serving of RF or a placebo (in a random double-blinded fashion). RF increased REE during initial measurement, and 1 and 2 h post-ingestion as well as average energy expenditure for the entire trial compared with the control treatment (Fig. 1a). These findings were observed using total energy expenditure and after controlling for lean body mass. RF consumption was associated with an average increase in daily REE of 159.7 ± 89.7 kcal/day. Surprisingly, RER was significantly elevated in the RF-treated trial compared with placebo treatment (Fig. 1b). Additionally, we measured the effects of RF consumption on resting blood pressure. RF treatment significantly elevated systolic blood pressure throughout the trial, however RF treatment did not alter diastolic blood pressure (Fig. 1c and d, respectively). 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 significant predictors of metabolic response to RF (data not shown).

3.2. *In vitro*

3.2.1. *RF enhances oxidative metabolism*

To investigate the molecular effects of RF on skeletal muscle, oxidative metabolism was measured in multiple myocyte cell models following treatment with various doses of RF for several durations. Treatment with RF for either 3 or 6 h significantly increased basal oxygen consumption in both cell models (Fig. 2a). Peak oxidative metabolism was also significantly increased in both cell models following 3 h of treatment, but was only significantly elevated in the C2C12 cell model following 6 h of treatment suggesting a differing response between the models with time-dependence (Fig. 2b). In correspondence with basal oxidative metabolism, cells treated with RF also exhibited significantly greater mitochondrial uncoupling (proton leak) (Fig. 2c).

3.2.2. *RF alters glycolytic metabolism and metabolic reliance*

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 massive increase in basal glycolysis compared with corresponding controls (Fig. 2d). 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 h. Conversely, C2C12 cells treated with RF showed significantly elevated peak glycolytic metabolism (Fig. 2e). Because cells were cultured 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 our group has previously described.[24] Despite discrepancies 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 (Fig. 2f).

3.2.3. *RF induces metabolic gene expression*

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 h. RD cells treated with RF exhibited significantly elevated PGC-1α expression at initial time points (Fig. 3a) while C2C12 cells only exhibited elevated PGC-1α expression following 4 h of treatment (Fig. 3b). RF also significantly induced NRF-1 and TFAM expression, downstream targets of PGC-1α, in both cell models. Additionally, GLUT4 expression was elevated above control levels at various time points for both cell models the most notable of which occurred at 24 h. In addition to heightened metabolic gene expression in RF treated cells, RF treatment was also associated with significantly elevated mitochondrial content in both cell models, measured by both flow cytometry and confocal microscopy (Fig. 3c 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 (Fig. 3e).

4. Discussion

Few published data are available regarding the effects of RF (or similar dietary supplements) 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 greater basal oxidative and total metabolism accompanied by a significant increase in peak oxidative capacity. Our data also demonstrate that RF significantly induced metabolic gene expression including PGC-1α, which was accompanied by a significant increase in mitochondrial content in both cell models. An

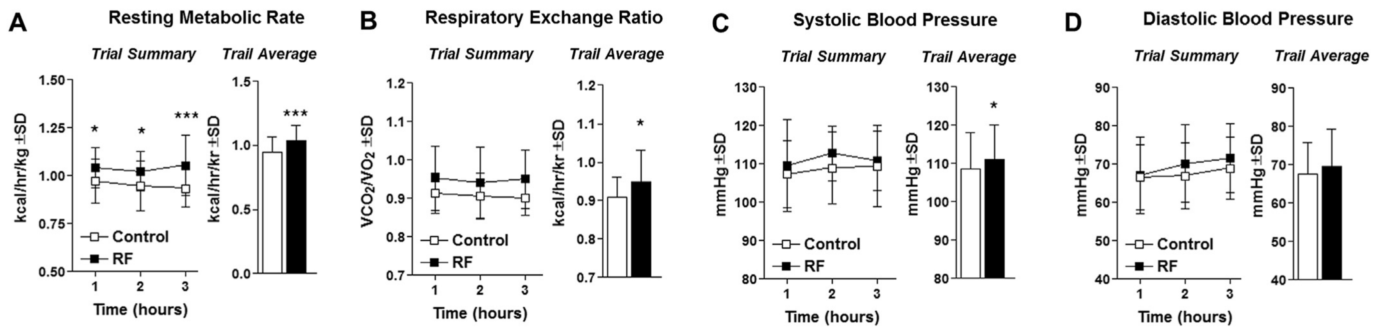

Fig. 1. Metabolic Rate and Substrate Utilization. (A) Resting energy expenditure (REE) of human male subjects following ingestion of either a placebo (control) or a single serving of RF. (B) Respiratory exchange ratio (RER) of human male subjects following ingestion of either a placebo (control) or a single serving of RF. (C) Systolic blood pressure of human male subjects following ingestion of either a placebo (control) or a single serving of RF. (D) 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.

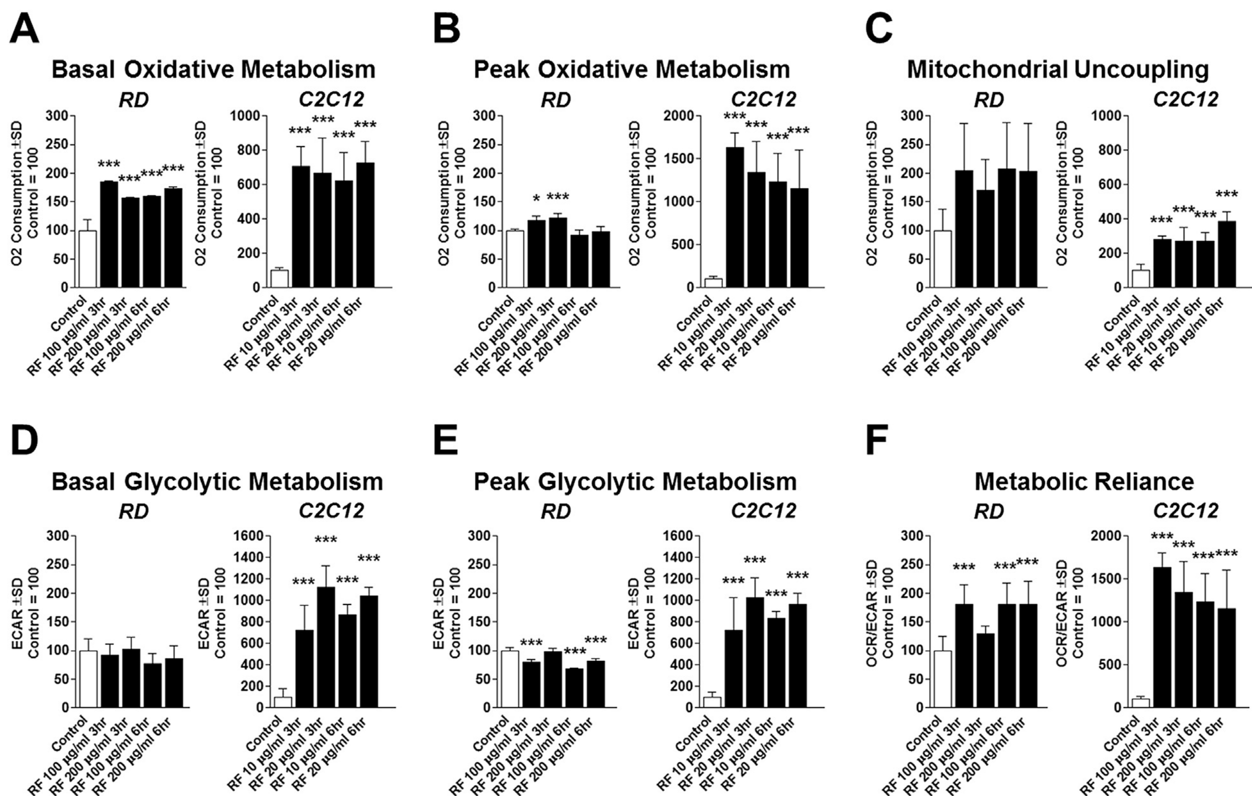

Fig. 2. Cellular Metabolism. (A) Basal oxidative metabolism indicated by oxygen consumption rate (OCR) of RD (left) cells treated with either ethanol control or RF at 100 μg/ml or 200 μg/ml for 3 or 6 h and C2C12 (right) cells treated with either ethanol control or RF at 10 μg/ml or 20 μg/ml for 3 or 6 h. (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 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.

interesting observation is that RD cells tolerated ten times greater concentration than the non-malignant myocytes, suggesting that RD cells are substantially more resilient. Moreover, the cancerous phenotype that RD cells possess (causing RD cells to preferentially select glycolytic metabolism over oxidative metabolism) may also explain the differential response in glycolytic metabolism between the cell models. These observations highlight potential issues (including cell model discrepancies) with experiments using *in vitro* observations alone.

The discrepancy between cell model ATP response is a meaningful observation because reduced cellular ATP content is a potent stimulus for cells and is likely in part responsible for the heightened metabolic activity with related gene expression. Not surprisingly, non-malignant C2C12 myocytes exhibited significantly reduced ATP content and a larger relative increase in both oxidative and glycolytic metabolism. One limitation of this work is that the specific upstream signaling targets of PGC-1α were not evaluated. However, the effect of caffeine and reduced cellular ATP content on 5-adenosine monophosphate-activated protein kinase (AMPK) activation are well documented, making AMPK a strong candidate responsible for increases in energy expenditure and PGC-1α expression.[32,33] Additionally, because oxidative metabolism is more

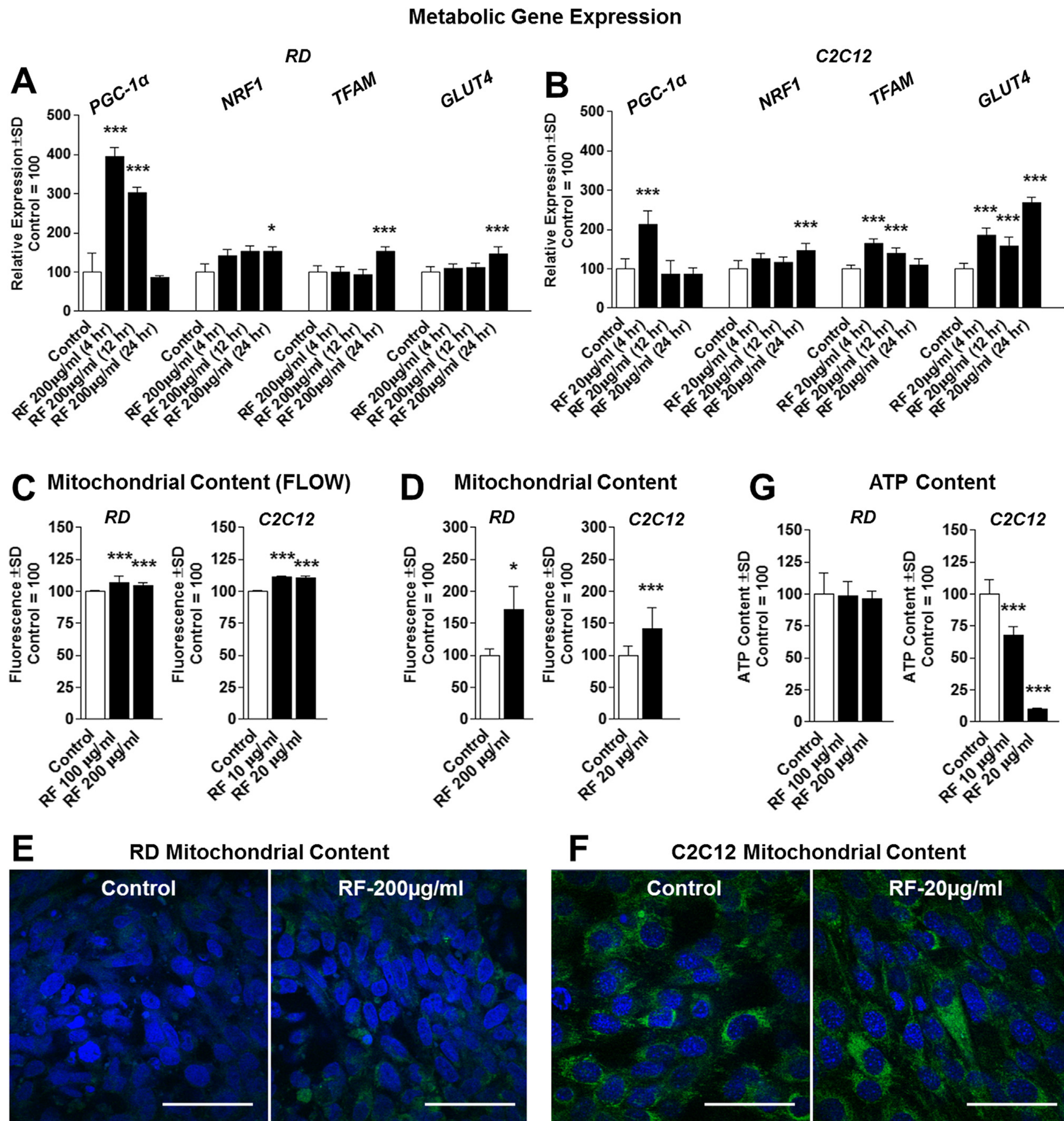

Fig. 3. Metabolic Gene Expression. (A) Gene expression of RD cells treated with either ethanol control or RF at 100 μg/ml or 200 μg/ml for 4, 12, or 24 h of PGC-1α, NRF-1, TFAM, and GLUT4. (B) Gene expression of C2C12 cells treated with either ethanol control or RF at 10 μg/ml or 20 μg/ml for 4, 12, or 24 h of PGC-1α, NRF-1, TFAM, and GLUT4. (C) Mitochondrial content indicated by group mean log fluorescence from flow cytometry of RD (left) and C2C12 cells (right) treated as described in methods for 24 h stained with MitoTracker green. (D) Group mean log fluorescence of confocal microscopy of RD cells treated as previously described for 24 h. (E and F) Representative images from fluorescent confocal microscopy of RD cells (E) and C2C12 cells (F) treated as described above. Fluorescent measurements were performed using $N = 7$ cells/treatment which were stained with MitoTracker (green) and DAPI (blue). (G) Cellular ATP content following treatment of either RD cells (left) or C2C12 cells (right) with RF at various doses for 24 h. NOTES: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ compared with control. White measurement bar represents 50 μm.

efficient at ATP biosynthesis, increased ATP consumption (or reduced efficiency of oxidative ATP production) is a necessary effect of potential anti-obesity agents. Our data suggests that RF reduces ATP production in part by promoting mitochondrial uncoupling. Despite this observation, it remains unclear if RF-induced mitochondrial uncoupling is sufficient to compensate for increased ATP production from oxidative metabolism. 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.[19,34,35]

In agreement with previous *in vivo* observations using similar dietary supplements, RF appears to increase metabolic rate in

young and healthy adult male subjects.[16–18] Although RF treatment elevated energy expenditure by approximately 160 kcals/day (as estimated from our 3 h measurements), this estimate may be inflated due to the time points measured (measurements closer to initial consumption yield greater increases in metabolism) and provide negligible benefit for weight loss. Several similarities exist between supplements designed to stimulate metabolism including stimulants such as caffeine which have previously been documented to increase metabolism in humans.[4,5,7,9,36] We have recently discussed many of these similarities including ingredient composition and effect on resting energy expenditure.[37] Despite these findings, it is unclear to what extent energy expenditure is increased for the purpose of weight loss. Because our measurements in human subjects were limited to 3 h following consumption, several questions remain unanswered about the effects of RF. First, at what point after a single dose does resting energy expenditure return to normal? Are these effects diminished with ongoing use (sensitization)? Are these effects also seen in older adults and/or women? Will increases in systolic blood pressure resolve with use? Another primary limitation of our work is that it is unclear if RF provides any metabolic benefit beyond that previously documented by caffeine or catechin consumption. Another limitation is that this study only measured the metabolic consequences of RF consumption during resting state, however many of the metabolic benefits of caffeine occur as a result of increased muscular activity, suggesting our findings may not reveal the complete metabolic effect(s) of RF during activities of daily living. Despite these and other limitations, our data adds to the current literature which supports the hypothesis that dietary composition of select food chemicals 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 current information difficult for consumers to trust. There currently exists an immense interest in both private and commercial sectors for exploring extracts (such as green tea (綠茶lǜ chá)) and other food chemicals for the benefit of metabolic diseases including obesity and diabetes supporting the need for ongoing research in dietary supplements and functional food chemicals.[36]

5. Conclusion

Dietary supplements are generally regarded as ineffective, under-researched, and potentially risky by the medical community. Despite this consensus, increasing consumer interest in alternative methods to increase metabolism and fat loss warrants further investigation into dietary supplements and thermogenic agents. Our observations suggest RF may be effective at increasing metabolic rate *in vivo* and *in vitro*; although from the current data it is doubtful RF provides benefits beyond those provided by natural dietary sources of ingredients found in RF. It is also unclear if regular consumption of RF will cause significant weight/fat loss in healthy subjects. Further research is needed to elucidate the full effects of habitual RF consumption on human metabolism and health.

Conflicts of interest

All authors have none to declare.

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