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# Fucoxanthin and its Metabolite Fucoxanthinol Do Not Induce Browning in Human Adipocytes

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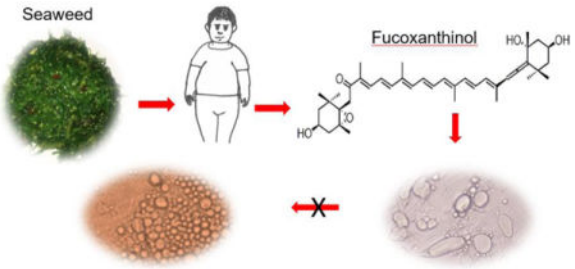
## Abstract

Rodent studies suggest that the anti-obesity effects of fucoxanthin relate to the activation of brown fat and the conversion of white adipocytes to the brown phenotype. To determine the browning effect in human adipocytes, we investigated the genes involved in browning and measured oxygen consumption rate (OCR). Data were analyzed by one way ANOVA. Relative to control, fucoxanthinol (1μM, 0.1μM, 0.01μM, 1nM, 0.1nM) the metabolite present in human plasma, stimulated lipolysis acutely (mean ±SEM: 4.2±0.8, 3.1±0.6, 4.1±0.9, 3.8±0.7, 3.8±0.7 respectively, $p<0.01$). There was no effect on OCR or the mRNA expression of UCP1, CPT-1β, and GLUT4, the genes associated with browning of adipose tissue, when human adipocytes were treated with fucoxanthin or fucoxanthinol. Gene expression of PGC-1α, PPARα, PPARγ, PDK4, FAS, and the lipolytic enzymes was not significantly altered by fucoxanthinol treatment ($p > 0.05$). Thus, in human adipocytes, fucoxanthin and its metabolite do not stimulate conversion of white adipocytes to the brown phenotype.

## TOC Image




### Keywords

Fucoxanthin; Fucoxanthinol; Adipocytes; Browning; Lipolysis

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## Introduction

Obesity is a multidimensional problem with complex contributing factors. Moreover, weight loss induces neuroendocrine changes that make it particularly difficult for individuals to adhere to diet regimens. In the short term a motivated individual will lose weight; but, weight maintenance is largely precluded by a biological resistance to weight loss and a predisposition to weight gain driven by counter-regulatory mechanisms that prompt an unconscious impulse to eat.[1] The need to exercise a constant control over eating behavior in the face of physiologic pressures to regain weight can be trying for even the most motivated individuals who have achieved weight loss. Thus, the importance of research focused on increasing energy expenditure and fat oxidation as a means of addressing obesity and its co-morbidities cannot be overstated.

The discovery that humans possess functional depots of brown adipose tissue (BAT)[2–4] sparked a fervent search for molecular targets that activate BAT and promote the conversion of white adipose tissue (WAT) to BAT, aptly called ‘beige’ or ‘brite’ adipose tissue. The accumulation of beige adipocytes in WAT, referred to as browning, has gained a lot of attention because BAT is typically low in humans and correlates inversely with body mass index.[4–6] When activated, triglycerides stored in BAT or beige adipose tissue are broken down and the chemical energy is dissipated by channeling fatty acids into beta oxidation.[7] Browning is characterized by increased expression of uncoupling protein-1 (UCP1) which uncouples electron transport from ATP production leading to controlled exothermic resolution of the proton gradient and the generation of heat. These processes increase energy expenditure.[8] In this type of metabolically active tissue glucose uptake is also stimulated.[8]

While the processes determining the fate of most cells are under strict regulation by genetic and internal signals, the fate of adipocytes is uniquely distinct in that nutrition and environment are the predominant factors that determine whether the cell will acquire a beige or white phenotype.[9]

Fucoxanthin is a carotenoid present in the chloroplasts of brown seaweed. Dietary fucoxanthin is mostly absorbed as fucoxanthinol, a hydrolyzed metabolite, from the small intestine and enters systemic circulation through lymph.[10] Fucoxanthin is known to reduce body weight and improve glucose metabolism in rodents fed a high fat diet, in part by increasing lipolysis and fat oxidation.[11–12] Although the results are not completely consistent, fucoxanthin has been shown to stimulate the induction of UCP1 in white adipose tissue of mice.[12–15]

In humans, fucoxanthin supplementation reduced body weight and hepatic lipid accumulation, increased energy expenditure, and had a positive impact on components of the metabolic syndrome.[16] However, the mechanism by which fucoxanthin exerts its physiologic effects on obesity and glucose metabolism remains unclear. The effects of fucoxanthin could be a result of increased browning of white adipose tissue and increased lipolysis with increased fat oxidation. Evidence from rodent studies cannot always be corroborated in humans; nevertheless, the added evidence from the results of the human trial prompted the hypothesis that fucoxanthin treatment would stimulate the browning of human

white adipocytes. Since fucoxanthin metabolites accumulate in the adipose tissue of mice,[17] this study investigated the effects of fucoxanthin and its metabolite fucoxanthinol on the browning of white adipose tissue, in primary human adipocytes.

## Materials and Methods

### Chemicals

Hyclone™ Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12 1:1) and Gibco™ fetal bovine serum (FBS) were purchased from ThermoFisher Scientific (Waltham, MA), Rosiglitazone was purchased from AK Scientific (Union City, CA). All other reagents used in the medium were purchased from Sigma-Aldrich (St. Louis, MO). Fucoxanthin (> 95% fucoxanthin, FN), bovine serum albumin (BSA), glycerol standard solution, and taurocholic acid were purchased from Sigma-Aldrich. Glycerol Reagent was purchased from Zenbio (The Triangle, NC). Isoproterenol was purchased from Cayman Chemicals (Ann Arbor, MI). An organic extract of the fucoxanthin plant containing 5wt% fucoxanthin (FEX) was provided by PLT Health Solutions, (Morristown, NJ). The purified alcohol of fucoxanthin, fucoxanthinol (FOL), was purchased from Wako Pure Chemical Industries (Osaka, Japan, 94% fucoxanthinol) and Sigma-Aldrich (≥ 97% fucoxanthinol). Cholesterol esterase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Protease and phosphatase were purchased from Cell Signaling Technology (Danvers, MA), RIPA buffer from Sigma, TGX protein gels from BIO-RAD (Hercules, CA).

### Methods

**Enzymatic Extraction—**To convert fucoxanthin in the plant extract to the biologically active form fucoxanthinol, the extract was subjected to enzymatic hydrolysis by cholesterol esterase. Fucoxanthin extract (FEX) at a concentration of 50μM (based on wt% fucoxanthin in the extract) was added to a solution containing 3ml Potassium Phosphate Buffer (0.1M, pH 7.0), 30.2 mg taurocholic acid and 2.1 mg cholesterol esterase and incubated for 4 hours in a rotating incubator at 37°C. This enzymatic hydrolysate (FEX-H) was partitioned and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis.

**Liquid chromatography–mass spectrometry—**Samples of FOL and FEX-H were analyzed by a LC-MS system consisting of Dionex UltiMate™ 3000 UPLC including Dionex HPG-RS pump, RS autosampler, RS column compartment, and Dionex UltiMate (ThermoFisher Scientific) photodiode array detector (PDA). After PDA the sample flow was guided to a Q Exactive™ Plus orbitrap (ThermoFisher Scientific) high resolution, high accuracy mass spectrometer. The LC mobile phase consisted of solvent A – 100% acetonitrile, and solvent B – 0.5% acetic acid in deionized, reverse osmosis filtered water. All LC separations were performed in isocratic mode – 50% solvent A and 50% solvent B. The mobile phase flow rate was 0.2 ml/min. Compounds were separated on a Phenomenex® Kinetex RP C8, 2.6 μ particle size, 100 × 2.1 mm column. The Q Exactive™ plus mass spectrometer was equipped with an electrospray ionization source (+ESI) operated in positive mode. The scan type was full MS from 150 to 2000 daltons. In arbitrary units, the sheath gas flow rate was 30, auxiliary gas flow was 8, and sweep gas flow rate was 1. The

spray voltage was 3.8 kV, capillary temperature was 320 °C, and auxiliary gas heater temperature was 380 °C. LC-MS analyses were performed at Rutgers University.

**Cell culture—**Human adipose-derived stem cells from overweight and obese female donors were purchased from LaCell, LLC (New Orleans, Louisiana). Preadipocytes were seeded in DMEM/F12 1:1 supplemented with 10% FBS and 1% antibiotic (penicillin/streptomycin/amphotericin). The medium was replenished every two days. One day following confluence, cells were differentiated for four days in media containing 70% DMEM and 30% DMEM/F12 1:1 supplemented with 3% FBS, 1% antibiotic, 1μM dexamethasone, 33uM biotin, 0.1μM insulin, 20μM pantothenate, 5μM rosiglitazone and 500μM 3-isobutylmethylxanthine. Following four days of differentiation, cells were maintained in medium composed of 70% DMEM and 30% DMEM/F12 1:1 supplemented with 3% FBS, 1μM dexamethasone, 33μM biotin, 0.1μM insulin, and 20μM pantothenate (day 0). Cells were kept at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$.[18]

**Treatments—**Treatments were started from day 3 to day 6 and assays were performed on day 10 to day 13. Treatments were administered every two days and dexamethasone was removed from the medium during treatment to promote browning of adipose tissue.[19] For evaluation of the acute effects, treatments were administered four hours prior to the assay. Based on a pharmacokinetic study in humans suggesting that 0.1μM fucoxanthinol was close to being attainable in human plasma following oral intake of fucoxanthin extract,[20] the study investigated the effects of treatment with 0.1μM and ten-fold concentrations above and below it.

**Lipolysis—**Adipocytes were grown in 96 well plates and cells were washed twice with Krebs-Ringer bicarbonate buffer (KRB). Treatments added to KRB + 2% BSA were given to the cells. Following a four hour incubation period, 50μl from each well was transferred to a corresponding well of a 96-well plate containing 50μl of glycerol reagent. Glycerol release was quantified by absorbance (540 nm, Versamax™ tunable microplate reader, Molecular Devices, Radnor, PA) after 15 minutes. Treatments included FN, FEX, and FEX-H at 1μM and 0.1μM concentrations. FOL stimulated lipolysis at these concentrations and was therefore further investigated at 0.01μM, 1nM, and 0.1nM concentrations. Isoproterenol was used as a positive control.

Fucoxanthin is converted to fucoxanthinol in adipocytes of mice in 48 to 57 hours.[21] Therefore, an experiment was also conducted where the cells were treated with FEX at 0.1μM concentration and the second treatment with FEX was added to the medium after 72 hours to allow the adipocytes to convert fucoxanthin to fucoxanthinol, following which the assay was conducted.

**Oil Red O Staining—**An Oil Red O stock was prepared as previously described.[22] Adipocytes grown in 48 well plates were treated with FOL 1μM, and incubated for four hours. In accordance with the protocol previously described,[23] the medium was aspirated and the wells were rinsed with PBS. Following incubation for 10 - 15 min in a solution to fix the cells (10% formaldehyde in PBS), the wells were rinsed 5 times with tap water. The remaining water was aspirated, and the cells were incubated for 1 hour in Oil Red O solution

(0.3% in isopropanol). Following incubation, the stain was aspirated, and the cells were rinsed 5 times with tap water. After visual examination, cells stained with Oil Red O were dissolved in isopropanol and quantified by absorbance (520 nm) measurement.

**Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)—**Adipocytes grown in 12 or 24 well plates were treated every 48 hours over a period of six days and each treatment was given in replicates of three or four respectively. Treatments included FN, FEX, FEX-H, and FOL at 0.1μM concentration. On day seven 500μl Trizol reagent was added to lyse adipocytes and the contents of each well were homogenized. RNeasy Mini Kit (Qiagen, Germantown, MD) was used to isolate RNA following manufacturer's protocol. The RNA was quantified using Invitrogen Qubit RNA broad range assay kit and read using Qubit Fluorometer (ThermoFisher Scientific). Reverse transcriptase and PCR were conducted in one reaction with the reverse PCR primer priming cDNA synthesis using SuperScript® III Platinum One-Step Quantitative RT-PCR System with Rox from Invitrogen (ThermoFisher Scientific). Amplicons were designed to span an intron-exon junction to avoid amplification of genomic sequences. Primer and probe oligonucleotides for UCP1, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARα), carnitine palmitoyltransferase-1β (CPT-1β), glucose transporter type 4 (GLUT4), and peroxisome proliferator-activated receptor gamma (PPARγ) were purchased from Integrated DNA Technologies (Coralville, IOWA) and are provided in Table 1. Rosiglitazone was used as a positive control for browning of white adipocytes.[24]

Each sample RT-PCR assay was conducted in duplicate. In some experiments, mRNA was reverse transcribed with oligodT primers (Invitrogen Super Script® IV First- Strand Synthesis System, ThermoFisher Scientific) and qPCR was performed using SYBR® green PCR Master Mix (Applied Biosystems). Primer sequences for adipose triglyceride lipase (ATGL), carnitine palmitoyltransferase-1α (CPT-1α), fatty acid synthase (FAS), glycerol kinase (GK), hormone sensitive lipase (HSL), and pyruvate dehydrogenase kinase 4 (PDK4) obtained from Integrated DNA Technologies and designed using the PrimerQuest tool are provided in Table 2. Tolerance to the dose was assessed with FOL at 10μM concentration.

**Western Blots—**Cells treated with FOL at 1μM for six days were lysed in RIPA buffer containing a cocktail of protease and phosphatase. TGX SDS-PAGE gels (Any Kd™, BioRad) were used to separate 50μg of solubilized protein per sample. Following transfer, nitrocellulose membranes were probed overnight at 4°C with primary antibodies against AMP-activated protein kinase (AMPK; rabbit anti-AMPK, 1:2000, #2535S, Cell Signaling Technology) and phospho-AMPK (p-AMPK; rabbit anti-p-AMPK, 1:2000, #2532S, Cell Signaling Technology); Secondary antibody anti-rabbit HRP (Abcam 6781) was used to detect specific antibody-antigen complexes. Proteins were visualized by chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Waltham, MA).

**Mitochondrial Bioenergetics—**To study mitochondrial bioenergetics in human white adiopocytes, a SeaHorse XF24 Extracellular Flux Analyzer (SeaHorse Bioscience, Billerica, MA) was used. The human adipose-derived stem cells were seeded onto the SeaHorse XF24 V7 plate. The protocol for evaluating the bioenergetics parameters of the adipocytes as

previously described [25] was modified to include isoproterenol to stimulate fatty acid release.[26] Briefly, sequential injections of oligomycin (2 μM) to inhibit ATP synthase, isoproterenol (1μM), carbonyl cyanide-4-(trifluoromethoxy) phenyhydrazone (FCCP, 3 μM), to uncouple electron transport and allow for maximum electron flux through the electron transport chain, and antimycin A (1.5 μg/mL), to inhibit complex III were used to determine basal oxygen consumption rate (OCR), ATP-linked respiration, maximal and reserve respiratory capacity. Cells were treated for six days with FOL 1μM, rosiglitazone 0.1μM or were untreated controls. After treatments, cells were washed with DMEM, then 625 μl/well serum-free SeaHorse XF media, containing 5mM pyruvate was loaded onto the plates. Plates were equilibrated in a non-$CO_2$ incubator at 37 °C for 20 minutes before obtaining bioenergetics profiles.

**Statistical Analysis—**One way analysis of variance was used to analyze the primary outcomes which were differences from the control. The level of significance for the tests was controlled at the nominal 0.05 level. The Welch test was used to evaluate homogeneity of variances and the F-test was used to test the hypothesis that the means of two groups were equal. Outcomes are summarized as means ± SEM. All analyses were performed using SAS 9.4 (SAS Institute, Cary NC) except the mitochondrial bioenergetics results which were analyzed using GraphPad Prism Software (La Jolla, CA) and are reported as means ± standard deviation.

## Results

### Liquid Chromatography – Mass Spectrometry

The samples of fucoxanthinol produced through in vitro hydrolysis of fucoxanthin (FEX-H) and purchased commercially (FOL) were characterized through LC-MS. The main peaks corresponding to fucoxanthinol were identical as seen in the chromatograms for FEX-H and FOL. There were other minor components that differed between the samples. (Figure 1A and B).

### Lipolysis

Acute treatment (four hours) with FN, FEX, and FEX-H, at 1μM and 0.1μM did not stimulate lipolysis in human subcutaneous adipocytes. In Caco-2 human intestinal cells fuocoxanthin is hydrolyzed to fucoxanthinol during uptake by the cells, and in humans and mice fucoxanthinol but not fucoxanthin appears in plasma following ingestion of fucoxanthin.[10, 20] The effect of FEX on human adipocytes was tested following an incubation of 72 hours to facilitate conversion to fucoxanthinol, but no effect on lipolysis was observed. Treatment with FOL at concentrations ranging from 1μM to 0.1nM for four hours stimulated lipolysis (Figure 2A). Oil Red O staining of the cells showed that FOL treatment produced a trend towards reduction in the lipid content of the cells (Figure 2B).

### Gene Expression

In human adipocytes, PPARγ or PPARα agonists induce UCP1 and promote the browning of white adipocytes. Browning of adipocytes is accompanied by simultaneous induction of fatty acid esterification, lipolysis, and fat oxidation, in a futile triglyceride/fatty acid cycling.

Further, brown fat is characterized by increased insulin-stimulated glucose uptake and gene expression of GLUT4.[9] Thus, in our study, the browning of adipose tissue was evaluated by examining the mRNA expression of the genes that regulate these effects; however, treatment of human adipocytes with 0.1μM FN or FEX did not induce UCP1, GLUT4, or CPT-1β in white adipocytes. The efficacy of the FEX-H was then tested to determine whether it induces browning in human adipocytes in vitro. FEX-H at 0.1μM concentration also failed to consistently stimulate UCP1, CPT-1β, or GLUT4. Since lipolysis was stimulated by FOL, its effects on gene expression were more extensively assayed, but at 0.1μM concentration this compound failed to significantly affect mRNA expression of UCP1, CPT-1β, CPT-1α, GLUT4, PPARγ, PPARα, PGC-1α, ATGL, HSL, PKD4, GK, or FAS in human adipocytes (Figure 3A). Rosiglitazone treatment which was used as a positive control stimulated more than 100 fold increase in UCP1 in human white adipocytes (Figure 3B). At 10μM concentration, FOL was toxic to human white adipocytes and induced cell death within 48 hours.

### Western Blots

Treatment of adipocytes with FOL at 1μM concentration over a seven day period or a six hour period did not increase the phosphorylation levels of AMPK protein compared to untreated controls (Figure 4). Total AMPK levels were similar with all treatments.

### Mitochondrial Bioenergetics

The difference between basal OCR and oligomycin-linked OCR represents the amount of oxygen that is ATP-linked. The balance of the basal OCR is comprised of oxygen consumption arising from proton leak, and non-mitochondrial sources related mainly to the action of oxygenases.[27] In intact cells, UCP1 must be activated by fatty acids.[7] As shown in Figure 5A, FOL treatment did not increase basal OCR, or OCR that is inhibited by oligomycin. Further, the addition of isoproterenol to release fatty acids did not stimulate an increase in OCR, maximal or reserve OCR in the FOL treated cells compared to untreated controls. (Figure 5B).

## Discussion

There were no changes in gene expression relating to the browning of adipose tissue, fat oxidation, and glucose transport in vitro in primary human adipocytes treated with fucoxanthin or fucoxanthinol, the metabolite present in human plasma following ingestion of fucoxanthin. There was also no increase in the OCR of cells treated with FOL. The results suggest that fucoxanthin or its metabolite do not promote the conversion of white adipocytes to the more metabolically active beige or brite phenotype at concentrations that are physiologically attainable in humans and are not toxic to cells. However, FOL stimulates acute lipolysis in human adipocytes.

In obese mice fed a high fat diet, six weeks of supplementation with fucoxanthin decreased adipocyte size, body weight gain, and visceral fat pads without affecting food intake, suggesting an increase in energy expenditure. The mRNA expression and activity of lipogenic enzymes decreased concomitantly with an increase in fatty acid beta-oxidation and

an upregulation of UCP1 mRNA expression in WAT.[13] In addition to positive effects on body weight, body fat and hepatic lipid droplet accumulation in mice, nine weeks of supplementation with fucoxanthin was accompanied by beneficial effects on glucose homeostasis.[28] Similarly in another study, mice fed a high fat diet supplemented with fucoxanthin had reduced body weight gain which was accompanied by a decrease in lipid accumulation in the liver and a reduction in serum triglycerides.[29] For the most part, these anti-obesity effects were demonstrated in a host of studies in mice fed a high fat diet.[11–12, 15, 30]

In a clinical trial, obese women supplemented daily for 16 weeks with 2.4 mg of fucoxanthin delivered in pomegranate seed oil (Xanthigen™) lost body weight, body fat, liver fat, and had reduced serum triglycerides compared to women given the placebo. In a small subset of the study (three or four subjects per group), Xanthigen™ supplementation increased resting energy expenditure.[16] Although the overwhelming evidence from rodent studies and the purported mechanisms based on the human trial could not be substantiated in human white adipocytes, the results are far from inconsequential.

The nuclear receptor PPARγ which has a well-established role in promoting adipogenesis and fat oxidation is expressed at highest levels in adipose tissue.[31–32] In 3T3-L1 cells, during differentiation fucoxanthin at 10μM and 25μM concentrations as well as its metabolite fucoxanthinol at 2.5μM, 5μM, and 10μM concentrations reduced lipid accumulation, and at 2.5μM and 5μM concentrations, fucoxanthin as well as fucoxanthinol downregulated PPARγ protein levels.[21] During 3T3-L1 preadipocyte differentiation, treatment with Xanthigen™ decreased lipid accumulation and inhibited the protein expression of PPARγ.[33]

Adipose development involves a differentiation switch that activates the expression of a new set of genes followed by lipid accumulation. PPARγ is induced during differentiation and its expression is paramount for normal adipogenesis.[34–35] Thus, the anti-obesity effects of fucoxanthin were attributed to the downregulation of PPARγ that it produced.[21, 33] However, PPARγ has paradoxical effects on adipocyte biology and also acts to increase insulin sensitivity. Additionally, in mature human white adipocytes, activation of PPARγ or PPARα produce a molecular pattern such as the induction of UCP1, CPT-1β, and PGC-1α gene expression that suggests the acquisition of a brown phenotype.[24] Thus, PPARγ may need to be selectively modulated in a gene- and tissue-specific manner.[36] In our study, PPAPγ mRNA expression was not affected by FOL treatment in mature adipocytes.

Despite the increase acute lipolysis, change in the expression of the genes involved in lipolysis such as ATGL, HSL, and FAS, or an increase in the phosphorylation of AMPK was not observed in our study, after treatment with FOL for seven days. In both mice and humans, stimulation of β-adrenergic receptors in adipose tissue initiates lipolysis. During this process, adenylyl cyclase mediated increases in cAMP result in the activation of protein kinase A (PKA) which phosphorylates perilipin 1 (PLIN1) and HSL. Additionally in human adipocytes, natriuretic peptides acting through protein kinase G (PKG) phosphorylate the same targets as the β-adrenergic agonists do when acting through PKA, to stimulate lipolysis.[37] Phosphorylation of PLIN1 stimulates the release of comparative gene

identification-58 (CGI-58) which has a potent effect on the activation of ATGL.[38] Activation of ATGL is an important step in stimulating the lipolytic cascade. Hence, it is likely that stimulation occurs at the level of activation of some of these lipolytic enzymes rather than at the level of gene expression.

Unlike FOL, FEX-H did not have an effect on lipolysis. FOL and FEX-H have similar LC-MS profiles, but they are not exactly the same. Although the main peaks corresponding to fucoxanthinol were identical, FEX-H was prepared from an extract containing 5% fucoxanthin. Therefore, there were other components present that may have contributed to differences in the lipolytic activity of FEX-H and FOL.

In mature 3T3-L1 cells, fucoxanthin at 10μM concentration induced the phosphorylation of AMP-activated protein kinase (AMPK) and acetyl coenzyme A carboxylase (ACC), and increased the expression of CPT1α.[29] Xanthigen™ decreased the protein expression of FAS and activated AMPK signaling in 3T3-L1 cells.[33] AMPK through phosphorylation and inactivation of ACC promotes the uptake of fatty acids into the mitochondria which is the rate-limiting step in fatty acid β-oxidation. The fall in ACC reduces its product malonyl CoA, an inhibitor of fatty acid entry into the mitochondria. Fatty acid transport into the mitochondria is dependent upon the CPT1 system.[39] In our study phosphorylation of AMPK did not increase with FOL treatment of human adipocyte. The downstream effects such as induction of the CPT1 system at were also not supported by examination of the mRNA expression of CPT-1α or CPT-1β.

The triglyceride-fatty-acid cycle is a process in which free fatty acids produced during lipolysis are esterified back to triglycerides in a seemingly wasteful endeavor that has high energy costs.[40] In adipocytes GK is typically lacking which prevents adipocytes from generating glycerol-3-phosphate and recycling the glycerol in a futile cycling. Certain compounds such as the thiazolidinediones can stimulate GK gene expression and thereby promote the triglyceride-fatty-acid cycle.[41] Additionally, in human beige adipocytes, PDK4 which phosphorylates and inactivates the pyruvate dehydrogenase complex is upregulated, which shifts glucose metabolism towards glycerol-3-phosphate production, rather than oxidation.[24] However, in our study FOL did not induce the gene expression of PDK4 or GK in human adipocytes and an effort to establish possible futile triglyceride cycling as a mechanism by which fucoxanthinol may increase energy expenditure did not meet with success. Thus, in human adipocytes, fucoxanthinol does not appear to stimulate fatty acid oxidation or futile cycling of triglycerides. However, that does not preclude the channeling of fatty acids into β-oxidation in other metabolically active tissues such liver and skeletal muscle tissue which may be the subject of future investigation.

Microplate respirometry provides a method for measuring thermogenesis in cultured white adipocytes that have been treated with agents to stimulate browning. The bioenergetics profile widely assayed measures four respiration states. Basal OCR is first determined, followed by the addition of oligomycin to differentiate oxygen consumption used for ATP synthesis (coupled respiration) from basal proton leak (basal uncoupled respiration), maximal respiratory capacity by the addition of the uncoupling agent FCCP, and non-mitochondrial oxygen consumption using antimycin A to block the electron transport chain.

However, UCP1 is constitutively inactive until activated by fatty acids.[7] Additionally recent evidence [26, 42] suggests that fatty acids stimulate thermogenesis that is UCP1 independent. As suggested in these studies we modified the protocol to include isoproterenol following oligomycin to detect any possible mitochondrial uncoupling. Although FOL stimulates acute lipolysis there was no increase in basal OCR or basal proton leak in the FOL-treated cells compared to controls. The addition of isoproterenol to stimulate lipolysis and activate UCP1 or thermogenesis that is UCP1-independent also did not increase OCR compared to controls.

In mice, following oral administration of mixed micelle containing 160nM (0.105mg or 3.5mg/kg body weight) of fucoxanthin, the $C_{max}$ for fucoxanthinol was 132nM.[17] In humans, one seventh of this dose produced a plasma $C_{max}$ of 44.2nM.[20] Thus, the doses of fucoxanthin and fucoxanthinol used in the present experiments were maintained at concentrations close to what may be expected to occur in human plasma without being toxic to the cells. While in 3T3-L1 cells, no toxicity was observed in cells treated with fucoxanthinol at 10μM concentration for 120 hours,[21] in mature human adipocytes FOL at 10μM concentration induced cell death within 48 hours. Moreover, following oral administration of fucoxanthin to mice, amarouciaxanthin A, a liver metabolite of fucoxanthin was evident in the plasma; however, this metabolite was absent in human plasma following ingestion of an extract of kombu containing fucoxanthin at 0.52 mg/kg of body weight.[17, 20] These differences and the high doses of fucoxanthin or fucoxanthinol used in the rodent studies and 3T3-L1 cell line may explain the disparate results found in human adipocytes. It is also likely that fucoxanthinol may exert its physiologic effects by acting in concert with tissues other than adipose tissue.

Adrenergic control over BAT and beige fat is mediated by adrenergic receptors (ARs) especially the β3-AR in rodents, which are largely expressed in their mature adipocytes. The β1 AR in rodent adipocytes is necessary for proliferation of brown adipocytes. Thus, the β1- and β3-ARs are both needed for inducing the thermogenic response. Human adipocytes predominantly express β1- and β2-ARs and direct evidence of substantial contribution of the β3-AR to the thermogenic program is currently lacking. The α2-ARs which inhibit cAMP signaling inhibit BAT thermogenesis in rats. Human adipocytes display a higher expression of α2-ARs than rodents. The interplay between the relative expression or affinities of various ARs influences the browning of white adipocytes.[43] Further, multiple factors known to stimulate browning such as irisin which is expressed and secreted by skeletal muscle and adipocytes in rodents has little evidence for its relevance to human physiology.[44] These differences may account for the diverse effects of fucoxanthinol on browning in human and rodent species.

Activity of the enzymes involved in lipolysis and fat oxidation was not evaluated in this study which may limit the interpretation of the results. Nevertheless, based on the results of this study, especially the lack of induction of UCP1 gene expression or increases in oxygen consumption one may conclude that neither fucoxanthin nor its metabolite fucoxanthinol stimulate the browning of human white adipocytes at doses that may be expected to occur in human plasma without being toxic to the cells; however, fucoxanthinol induces lipolysis in human white adipocytes.

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## Abbreviations

| | |
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| **ATGL** | Adipose triglyceride lipase |
| **AMPK** | AMP-activated protein kinase |
| **BAT** | Brown fat |
| **BSA** | Bovine serum albumin |
| **CPT1-α** | Carnitine palmitoyltransferase-1α |
| **CPT-1β** | Carnitine palmitoyltransferase-1β |
| **FAS** | Fatty acid synthase |
| **FBS** | Fetal bovine serum |
| **FEX** | Fucoxanthin organic plant extract (PLT Health Solutions) |
| **FEX-H** | Enzymatic hydrolysate of fucoxanthin organic plant extract |
| **FCCP** | Carbonyl cyanide-4-(trifluoromethoxy) phenyhydrazone |
| **FOL** | Fucoxanthinol |
| **FN** | Fucoxanthin (Sigma Aldrich) |
| **GK** | Glycerol kinase |
| **GLUT 4** | Glucose transporter type 4 (GLUT4) |
| **HSL** | Hormone sensitive lipase |
| **LC-MS** | Liquid chromatography - mass spectrometry |
| **OCR** | Oxygen consumption rate |
| **PDK4** | Pyruvate dehydrogenase kinase 4 |
| **PGC-1α** | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| **PKA** | Protein kinase A |
| **PLIN1** | Perilipin 1 |

| | |
|---|---|
| **PPARα** | peroxisome proliferator-activated receptor alpha |
| **PPARγ** | Peroxisome proliferator-activated receptor gamma |
| **UCP1** | Uncoupling protein 1 |
| **WAT** | White fat |

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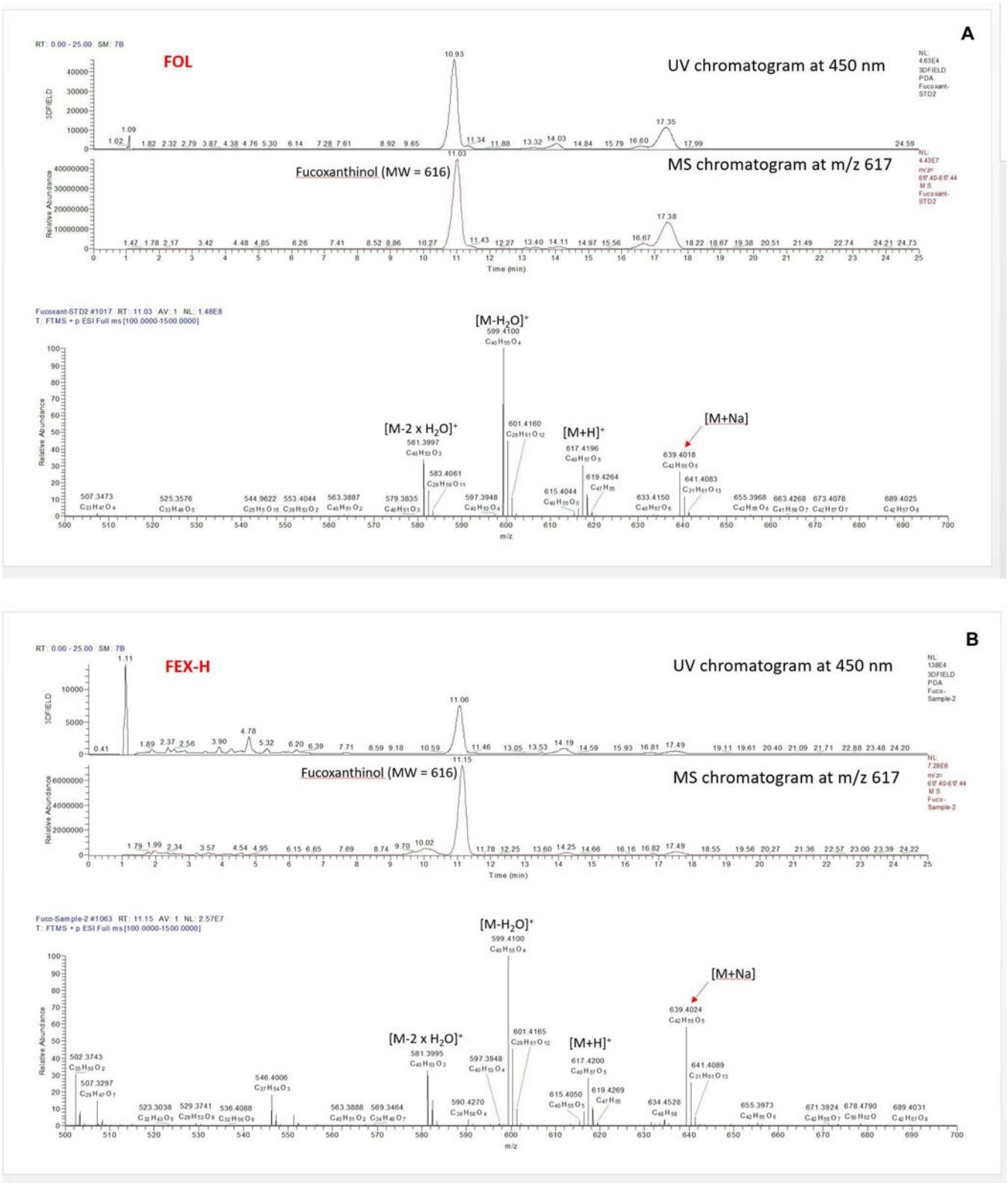


**Figure 1.**

Liquid chromatography - mass spectrometry (A) fucoxanthinol (FOL) purchased from Wako Pure Chemicals (Osaka, Japan) and (B) fucoxanthinol (FEX-H) obtained through in vitro enzymatic hydrolysis

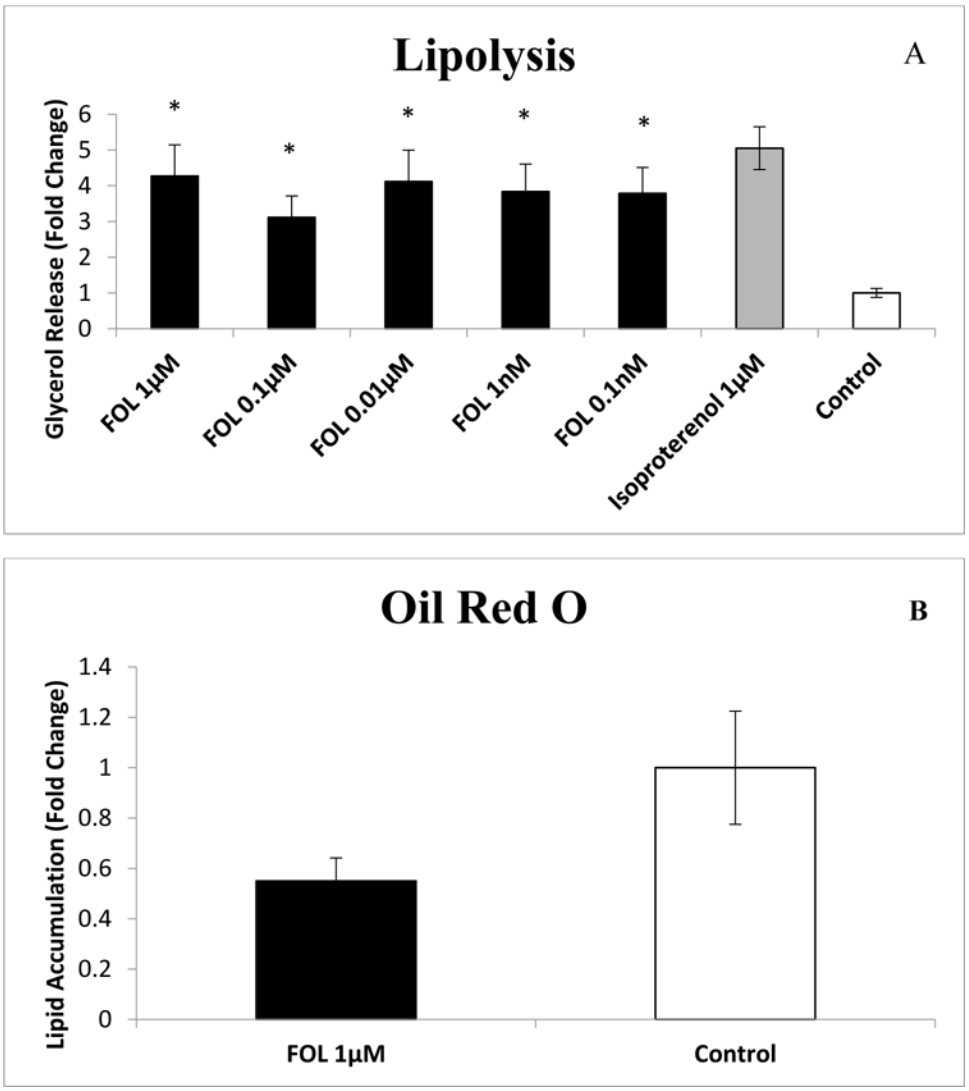


**Figure 2.**

(A) Glycerol release following treatment with fucoxanthinol (FOL) over a period of four hours. Outcomes are summarized as means ± SEM. *Shows statistically significant differences ($p < 0.01$). (n=16) (B) Adipocytes were grown in 48 well plates, treated with FOL 1µM and incubated for four hours (n=6, technical replicates =3). Cells were stained with Oil Red O and extracted by isopropanol. The lipid content was quantified by spectrophotometric analysis at 520 nm. There was a trend towards reduction of lipids in the cells treated with FOL compared to untreated controls, $p = 0.1$.

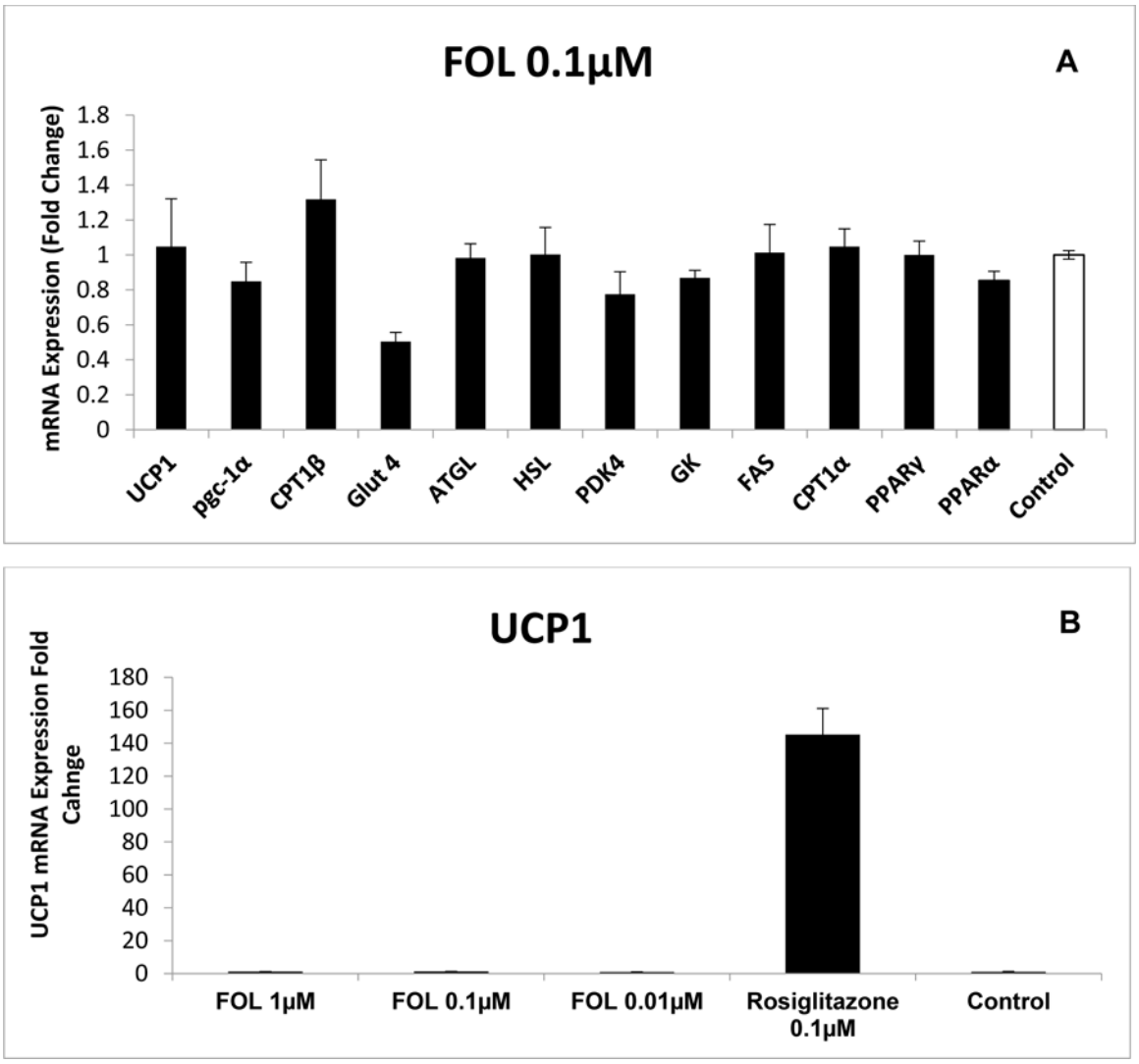


**Figure 3.**

RT-PCR for mRNA expression conducted in duplicates following treatment of human adipocytes with fucoxanthinol (FOL) at 0.1µM concentration for seven days did not demonstrate an activation of browning: (A) uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor gamma (PPARγ), peroxisome proliferator-activated receptor alpha (PPARα), carnitine palmitoyltransferase-1β (CPT-1β), glucose transporter type 4 (GLUT4), adipose triglyceride lipase (ATGL), carnitine palmitoyltransferase-1A (CPT-1α), fatty acid synthase (FAS), glycerol kinase (GK), hormone sensitive lipase (HSL), and pyruvate dehydrogenase kinase 4 (PDK4) (B) Rosiglitazone used as a positive control induced more than 100 fold increase in UCP1 but FOL did not have an effect on mRNA expression of UCP1, (n=4 for all except UCP1 (n=8), CPT1β (n=15), PPARγ (n=8), ATGL (n=11), $p > 0.05$.

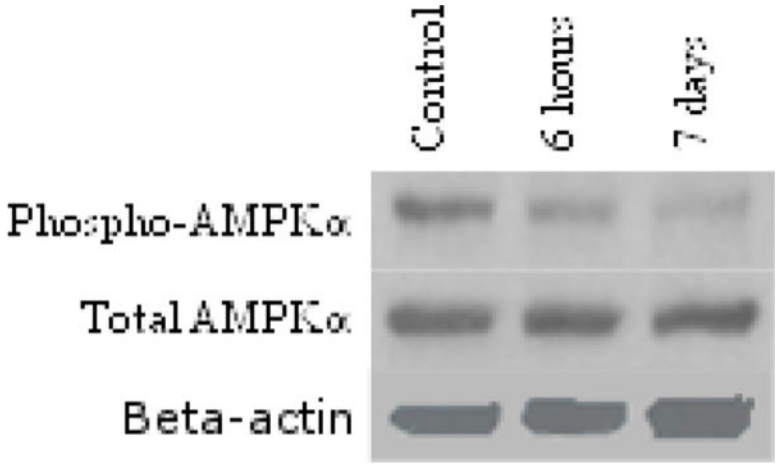


**Figure 4.**

Human adipocytes were treated with fucoxanthinol (FOL) at 1μM concentration for seven days or six hours. Cellular protein was extracted and Western blot analysis was used to detect phosphorylation levels of AMP-activated protein kinase (AMPK). FOL treatment did not increase the phosphorylation of AMPK.

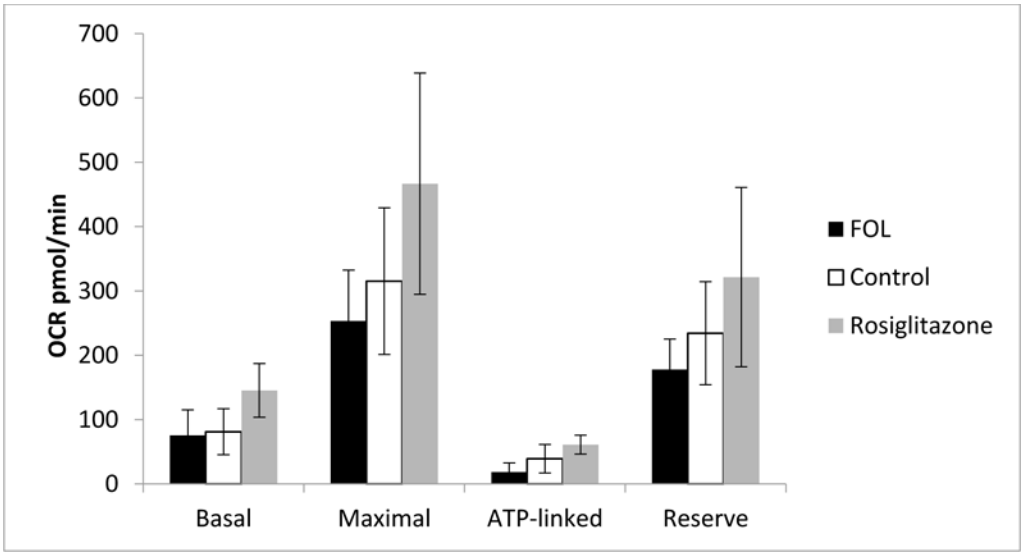


**Figure 5.**

Oxygen consumption rate (OCR) was measured in mature human adipocytes that were grown in the SeaHorse XF24 V7 plate and treated for six days with FOL at 1μM concentration (n=5), rosiglitazone 1uM (n=4) or were untreated controls (n=8). The cells were washed and changed to assay medium before assessment of mitochondrial function using oligomycin (2 μM), Isoproterenol (1μM), FCCP (3 μM), and antimycin A (1.5 μg/mL). Average OCR trace, depicting basal oxygen consumption, ATP-linked OCR after the addition of oligomycin, and maximal respiratory rate and reserve capacity calculated after the Isoproterenol and FCCP injection. Bioenergetic parameters were inferred from the OCR trace. The results are represented as mean ± standard deviation. Basal, ATP-linked, Maximal or Reserve OCR was not significantly different between the FOL-treated cells and the untreated controls ($p > 0.05$).

## Table 1

Primer and probe sequences for qPCR

| Gene | Primer and Probe | Sequences | Taqman ID |
|---|---|---|---|
| CPT-1β | Forward | TACCATGGGTGGATGTTTGAG | Hs00189258_m1 |
| | Reverse | GTCTGGAAGCTGTAGAGCATAG | |
| | Probe | TCTGGGCTATGTGTATCCGCCTTCTA | |
| GLUT4 | Forward | GTATCATCTCTCAGTGGCTTGG | Hs00168966_m1 |
| | Reverse | ATAGGAGGCAGCAGCATTG | |
| | Probe | AAAGGGCCATGCTGGTCAACAATG | |
| PGC-1α | Forward | CACCAAACCCACAGAGAACA | Hs01016719_m1 |
| | Reverse | GGGTCAGAGGAAGAGATAAAGTTG | |
| | Probe | AAAGAAGTCCCACACACAGTCGCA | |
| PPARα | Forward | GTCGATTTCACAAGTGCCTTTC | Hs00231882_m1 |
| | Reverse | CAGGTAAGAATTTCTGCTTTCAGTT | |
| | Probe | AACGAATCGCGTTGTGTGACATCC | |
| PPARγ | Forward | CCCAAGTTTGAGTTTGCTGTG | Hs00234592_m1 |
| | Reverse | GCGGTCTCCACTGAGAATAATG | |
| | Probe | TGGAATTAGATGACAGCGACTTGGCA | |
| UCP1 | Forward | GAGGAGTGGCAGTATTCATTGG | Hs00222453_m1 |
| | Reverse | CCGTGTAGCGAGGTTTGATT | |
| | Probe | TTCAAGCACAGAGCCATCTCCACG | |

Carnitine palmitoyltransferase 1B (CPT-1β), glucose transporter type 4 (GLUT4), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma (PPARγ),uncoupling protein 1 (UCP1)

**Table 2**

Primer sequences for qPCR using cDNA

| Gene | Direction | Sequences | NCBI Reference Sequence |
|---|---|---|---|
| ATGL | Forward | CGTGTACTGTGGGCTCATC | NM_020376.3 |
| | Reverse | GGACACTGTGATGGTGTTCTTA | |
| CPT-1α | Forward | TCCTGGTGGGCTACAAATTAC | NM_001876.3 |
| | Reverse | ACAGCAGATCCATGGCATAATA | |
| FAS | Forward | CATGGAGCGTATCTGTGAGAA | NM_004104.4 |
| | Reverse | GCTCATCGTCTCCACCAAA | |
| GK | Forward | GGTACTTCTTATGGCTGCTACTT | NM_203391.3 |
| | Reverse | GAACTGAGTGAGTCCACAGATTAT | |
| HSL | Forward | GACAGAGCTGGGATTTCTAACA | NM_005357.3 |
| | Reverse | AATCTGTGACCCACTCAGAAAG | |
| PDK4 | Forward | CGTGTATGTTCCTTCTCACCTC | NM_002612.3 |
| | Reverse | GGTGTAAGGGAAGGCTGATTT | |

National Center for Biotechnology Information (NCBI), Adipose triglyceride lipase (ATGL), carnitine palmitoyltransferase 1A (CPT-1α), fatty acid synthase FAS), glycerol kinase (GK), hormones sensitive lipase (HSL), and pyruvate dehydrogenase kinase 4 (PDK4)