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Metabolic inflexibility with obesity and the effects of fenofibrate on skeletal muscle fatty acid oxidation

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Abstract

This study was designed to investigate mechanisms of lipid metabolic inflexibility in human obesity and the ability of fenofibrate (FENO) to increase skeletal muscle fatty acid oxidation (FAO) in primary human skeletal muscle cell cultures (HSkMC) exhibiting metabolic inflexibility. HSkMC from 10 lean and 10 obese, insulin resistant subjects were treated with excess fatty acid for 24 hours (24hFA) to gauge lipid-related metabolic flexibility. Metabolically inflexible HSkMC from obese individuals were then treated with 24hFA in combination with FENO to determine effectiveness for increasing FAO. Mitochondrial enzyme activity and FAO were measured in skeletal muscle from subjects with pre-diabetes (n=11) before and after 10 weeks of fenofibrate in vivo. 24hFA increased FAO to a greater extent in HSkMC from lean *vs.* obese subjects (+49% *vs.* +9%, for lean *vs.* obese, respectively; $p<0.05$) indicating metabolic inflexibility with obesity. Metabolic inflexibility was not observed for measures of cellular resporiration in permeabilized cells using carbohydrate substrate. Fenofibrate co-incubation with 24hFA, increased FAO in a subset of HSkMC from metabolically inflexible, obese subjects ($p<0.05$), which was eliminated by PPARα antagonist. In vivo, fenofibrate treatment increased skeletal muscle FAO in a subset of subjects with pre-diabetes but did not affect gene transcription or mitochondrial enzyme activity. Lipid metabolic inflexibility observed in HSkMC from obese subjects is not due to differences in electron transport flux, but rather upstream decrements in lipid metabolism. Fenofibrate increases

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the capacity for FAO in human skeletal muscle cells, though its role in skeletal muscle metabolism in vivo remains unclear.

Keywords

mitochondria; fat oxidation; insulin resistance

Introduction

The capacity to modify substrate oxidation in response to changes in macronutrient availability is a hallmark of healthy metabolic tissues [1]. While many studies have investigated this metabolic flexibility in response to changes in glucose supply [2–4], fewer have examined the ability to adjust fatty acid oxidation (FAO) in response to fatty acid exposure. There is evidence of reduced mitochondrial capacity and FAO in the skeletal muscle of obese and/or diabetic humans 5–8], however, the underlying mechanisms linked with this metabolic inflexibility and interventions aimed increasing skeletal muscle FAO are not clearly evident.

We have previously reported a dampened ability to increase FAO in response to lipid exposure in skeletal muscle and primary human skeletal muscle cells (HSkMC) from obese, insulin resistant humans [6,9]. In addition, we observed an inability to upregulate peroxisome proliferator-activated receptor (PPAR)α and PPARα-responsive genes in response to high fat feeding in skeletal muscle of obese, insulin resistant humans [10]. PPARα is a global transcriptional regulator of FAO, activated by endogenous fatty acids and fatty acid derivatives [11]. Thus, PPARα is a prominent candidate for interventions aimed at increasing FAO and improving metabolic flexibility in obese, insulin resistant individuals who exhibit a reduced capacity for appropriately modulating FAO in response to nutrient availability.

Fibrates such as fenofibrate (FENO) are PPARα activators commonly used for treatment of hypertriglyceridemia [12]. These PPARα activators are known to induce the expression of genes involved in lipid oxidation, including pyruvate dehydrogenase kinase (PDK)4 and carnitine palmitoyl transferase (CPT)1 [13] in vitro or in animal models; however, there are limited data on whether FENO can increase FAO in metabolically inflexible, obese insulin-resistant humans. In addition, there are conflicting reports on effects of fibrates on insulin resistance [14–20]. Thus, as a regulator of PPARα, and potentially FAO, this series of experiments was designed to investigate the mechanisms linked with the lipid metabolic inflexibility of human obesity and the ability of FENO to increase FAO in human skeletal muscle.

Methods

HSkMC were derived from muscle biopsy specimens of lean and obese, insulin resistant humans and used to examine metabolid inflexibilty, FAO, and the effects of FENO. In vivo effects of FENO were determined in skeletal muscle samples collected before and after 10 weeks of FENO treatment in pre-diabetic individuals.

HSkMC

The East Carolina University Policy and Review Committee on Human Research approved this protocol and written, informed consent was obtained prior to the muscle biopsy procedure. For the HSkMC studies, skeletal muscle biopsies were performed as described [10,21] in the vastus lateralis of 10 lean (BMI<25 kg/m^2) and 10 obese (BMI>30 kg/m^2) Caucasian males (ages 18–31 y). Participants were free from disease, nonsmokers, and not taking medications known to alter lipid or carbohydrate metabolism. Subjects were excluded if they were performing more than 60 min/week of organized physical activity, or if they experienced a weight change greater than 2 kg in the previous 3 months.

Skeletal muscle satellite cells were isolated and cultured from fresh skeletal muscle tissue and differentiated to myotubes for 7 days [9]. Metabolic flexibility was tested by incubating cells for 24 h in differentiation medium supplemented with either 0.1% BSA (control; CTRL) or 200 μM oleate:palmitate (1:1 ratio) bound to BSA (2.5:1 molar ratio) plus 2 mM carnitine (24hFA), after which metabolic measures were made to determine: 1) FAO of intact HSkMC using ^{14}C-labeled fatty acid incorporation to $^{14}CO_2$; or 2) mitochondrial oxygen consumption of permeabilized HSkMC (Seahorse XF-24, Seahorse Bioscience, Billerica, MA).

Experiments were performed to determine the optimal FENO concentration and duration of incubation considering cell viability and capacity for increasing FAO (2h, 6 μM). In subsequent experiments, HSkMCs were exposed to 24hFA and 2h, 6 μM FENO in combination with 10 μM GW6471 (PPARα inhibitor), 20 μM Compound C (AMPK inhibitor), or 0.5 M AICAR (AMPK activator) to discern the mechanism of FENO action on HSkMC FAO.

HSkMC Fatty Acid Oxidation Assays

FAO assays were performed as described [22]. Briefly, myotubes were incubated at 37 °C in sealed 24-well plates containing differentiation media containing 0.25 μCi/ml [^{14}C]oleate and 0.25 μCi/ml [^{14}C]palmitate (PerkinElmer Life Sciences, Waltham, MA), with 200 μM, 1:1 ‘cold’ oleate:palmitate. After a 2h incubation, the rate of FAO was determined by measuring the $^{14}CO_2$ released from the media following acidification. Measures were performed in triplicate and data were corrected for total protein content, as measured by BCA method (bicinchoninic acid assay; Pierce Biotechnology, Inc.)

HSkMC Respiration Experiments

For permeabilized cell respiration, media was removed and 200 μl of sucrose-based respiration buffer (MiR05), supplemented with 2.5 μg/ml digitonin, were added. After 5 min, an additional 300 μl MiR05 respiration buffer containing basal substrate conditions (5 μM palmitoyl carnitine, 1 mM malate) but *without* digitonin supplement, were added. Cells were then subjected to the following respiration experiment: basal state 4 respiration (palmitoyl carnitine + malate, PCM_4), state 3 respiration supported by palmitoyl carnitine (+2 mM ADP; PCM_3), state 3 respiration supported by succinate (+3 mM succinate +1 μM rotenone; SR_3), state 2 respiration (+2 μM oligomycin; Olig.), and maximal uncoupled respiration (+4 μM FCCP). Respiratory control ratios (RCR)s were calculated as the state 3

respiration rate (succinate + rotenone) / state 4 respiration rates and were used to assess mitochondrial integrity. Following experiments, cells were rinsed with PBS, and lysed with protein lysis buffer, and total protein content was measured by BCA assay. All respiration data were normalized to cell-free wells and total protein content.

In Cell ELISA

HSkMC were rinsed twice in PBS then fixed to culture plates with 4% formalin for 5 min. Cells were permeabilized using permeabilization/blocking buffer (0.1% Fraction V BSA, 5% goat serum, 0.3% Triton X-100, 0.2% sodium azide in PBS) with rotation for 1 hr. After rinsing, 1:250 dilution of rabbit anti-AMPK or rabbit anti-phosphorylated AMPK [Thr172] in antibody buffer (0.1% Fraction V BSA, 5% goat serum in PBS) were added, then incubated overnight at 4°C with rotation. After rinsing. goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody diluted 1:5,000 in antibody buffer was added, then incubated at room temperature for 1 hr. Rinsed wells were emptied and blotted dry, HRP developing solution was added, and color change was measured at 1 min intervals for 10 min at 650 nM. Stable slopes for 5 consecutive minutes were used to quantify the content of phosphorylated AMPK relative to total AMPK (phosphorylated AMPK/total AMPK) protein.

Pre-diabetic Subjects and Study Design

To study the effects of FENO on in vivo skeletal muscle metabolism, muscle biopsies were obtained from individuals (age 18–65 y, BMI 28–38 kg/m^2) with pre-diabetes (defined as impaired glucose tolerance [IGT; plasma glucose of 140–199 mg/dl 2 h after a 75 g oral glucose tolerance test] *or* impaired fasting glucose [IFG; fasting plasma glucose of 100–126 mg/dl]) recruited as part of a randomized clinical trial () [21]. All subjects provided written, informed consent under a protocol that was approved by the local Institutional Review Board. Studies were conducted at the Clinical Research Center at the University of Colorado Anschutz Medical Campus.

Subjects with a history of diabetes, renal insufficiency (creatinine >1.4), liver disease (AST or ALT> 2x normal), congestive heart failure, or coronary artery disease were excluded, as were subjects who were taking medications known to affect inflammation or insulin resistance, such as steroids or NSAIDS. Baseline measures included insulin resistance by insulin-modified, frequently sampled intravenous glucose tolerance tests (FSIGT) and body composition by dual-energy X-ray absorptiometry (DXA) followed by vastus lateralis skeletal muscle biopsies (n=11). Subjects then received FENO (145 mg/day) for 10 weeks and FSIGT, DXA, and muscle biopsy repeated.

Fasting insulin was measured using an immunochemiluminescent assay (MLT Assay, Wales, UK). Plasma glucose was measured in duplicate by a glucose oxidase assay. Insulin sensitivity was calculated from the insulin and glucose data using the MinMod Millennium program [23].

Skeletal Muscle Fatty Acid Oxidation

Skeletal muscle FAO was measured as for HSkMC with modification for tissue homogenates as previously described [24] (n=3). Tissue homogenates were assayed for total protein content by BCA assay for data normalization. All measures were performed in quintuplet.

Mitochondrial Enzyme Activity

Mitochondrial-enriched supernatants (post 600 g) were prepared from frozen skeletal muscle samples [25]. Supernatants were used to assay activity of respiratory chain enzyme complexes I and II+III, and citrate synthase (CS) spectrophotometrically on a Synergy H1 microplate reader (Biotek, Winooski, VT). For complexes I, II+III, and CS enzyme activities were calculated as initial rates (nmol/min). For complex III, enzyme activity was calculated as the first-order rate constants derived within 2–3 min of reaction initiation. The protein content of each sample was determined by BCA assay. All enzyme activities were normalized to the total protein content of each sample and results are expressed relative to CS activity.

Quantitative PCR

Total RNA was isolated from skeletal muscle tissue using the Ultraspec RNA kit (Biotecx Laboratories, Inc, Houston, TX). cDNA was transcribed from 200 ng total RNA using iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed using primer sets for genes of interest and RPL13a and ubiquitin C as reference genes and iQ SYBR Supermix (Bio-Rad, Hercules, CA) as described [25]. Reactions were run in duplicate on an iQ5 Real-Time PCR Detection System (Bio-Rad) along with a no-template control per gene. RNA expression data was normalized to reference genes using the comparative threshold cycle method.

Mitochondrial DNA Copy Number

Total cellular DNA was isolated by phenol/chloroform extraction and quantified using the PicoGreen DNA quantification kit (Thermo Fisher, Waltham, MA). mtDNA content was measured as relative copy number of mtDNA per diploid nuclear genome using quantitative PCR with primers specific to β-globin and mtDNA, as described [26,27].

Statistical Analyses

For all characteristic data, independent *t*-tests were used. For HSkMC comparisons of 24hFA across body size, repeated measures ANOVA was used to test for effect of BMI, effect of treatment, or for interaction. For HSkMC FENO concentration/time course experiments, three separate univariate analyses of variance (ANOVAs) were used to compare across 24hFA pre-treatments and across FENO concentrations. Bonferroni corrections were used for *post-hoc* analyses. Separate independent *t*-tests were used to compare 24hFA with all other treatments for FENO mechanism experiments. Paired *t*-tests were used to test FENO treatment effects in human skeletal muscle tissue experiments. Statistical difference is indicated at $p \leq 0.05$. Data are expressed as the mean ± SEM. Statistical analyses were performed using SPSS Statistics (SPSS, IBM Corp., Armonk, NY).

Results

HSkMC subject characteristics

The characteristics of lean and obese subjects for the HSkMC experiments are presented in Table 1. By design, the obese group had a higher BMI and body fat percentage. While fasting glucose levels were similar between groups, the obese individuals had higher fasting insulin levels and HOMA-IR scores ($p<0.05$), suggesting insulin resistance.

Responses to 24h lipid incubation

FAO increased with 24hFA ($p<0.05$), though the lean subjects exhibited a 4-fold greater response to 24hFA than obese (+40% *vs.* +9% from CTRL to 24hFA conditions in lean *vs.* obese, respectively; Fig. 1*A*; $p<0.05$), indicating greater metabolic flexibility. We did not observe differences in mitochondrial content, as estimated by mitochondrial DNA copy number (Fig. 1*B*). To further investigate the pathogenesis of metabolic inflexibility, mitochondrial function was evaluated in similar conditions in permeabilized HSkMCs. Notably, in permeabilized HSkMC, state 3 respiration supported by palmitoyl carnitine, or PCM_3, was increased with 24hFA conditions in lean subjects but *decreased* in HSkMC from obese individuals (+34% and −17%, respectively; Fig. 1*B*, *inset*; $p<0.05$). However, mitochondrial respiration in all other conditions tested were not different between groups, nor did respiration change with 24hFA in either group for SR_3, Olig, or FCCP (Fig. 1C). Mitochondrial respiratory control ratios (mean = 2.78 ± 0.26) and state 4 respiration (Fig. 1C) were similar between groups and not altered with 24hFA.

Acute FENO treatment increases FAO

Acute (2h) FENO incubation stimulated a 4 to 5-fold increases in FAO for both the CTRL and 24hFA conditions in a dose dependent manner (Fig. 2A; $p<0.05$). A 12 μM FENO treatment also increased FAO to a similar magnitude as the 6 μM FENO, though this condition resulted in considerable cell death (data not shown). Longer duration FENO incubations were not effective for increasing FAO (Fig. 2B). Longer duration FENO incubations were also performed for 3 μM and 12 μM FENO concentrations, with no significant increase in FAO (data not shown). Thus, 2h, 6 μM FENO was chosen for subsequent experiments.

FENO may act through PPARα and AMPK phosphorylation

To test the specificity of the effect of FENO on FAO, we used GW6471 (a PPARα antagonist) or compound C (an AMPK inhibitor). FENO-mediated increases in FAO, were abolished with GW6471 co-incubation (Fig. 3A; $p<0.05$), indicating that FENO-mediated increases in FAO depend on PPARα activity. Likewise, co-incubation of FENO with Compound C, a competitive inhibitor of AMPK, abolished the effect of FENO on FAO and reduced FAO to below basal rates (CTRL or 24hFA alone; Fig. 3A, $p<0.05$). To corroborate these metabolic effects, Fig. 3B shows that AMPK phosphorylation was increased in the cells treated with 24hFA + 2h, 6 μM FENO ($p<0.05$), to a similar extent as observed with 0.5 μM AICAR ($p<0.05$). These effects of FENO were eliminated when GW6471 or Compound C were added (Fig. 3B).

Effects of FENO in individuals with pre-diabetes

Baseline characteristics and clinical outcomes of the 10-week FENO treatment in obese, pre-diabetic patients are summarized in Table 1. As expected, FENO treatment lowered triglyceride levels by 45% ($p<0.05$). However, insulin sensitivity, when measured by either HOMA-IR or FSIGT, did not change. There were no differences in citrate synthase activity (Fig. 4A), mitochondrial ETS enzyme activity (CI, CII+CIII, or CIV; Fig. 4B), or in mRNA content of PPARα-responsive genes (Fig. 4C). Nevertheless, in a subset of subjects, FAO of fresh skeletal muscle tissue homogenates increased by 10–80% from Pre- to Post-FENO treatment ($n=3$, Fig. 4D, $p<0.05$).

Discussion

Metabolic flexibility, defined as the ability to appropriately adjust oxidation to changes in nutrient availability, is a hallmark of metabolic health. In skeletal muscle specifically, metabolic flexibility is related to higher insulin sensitivity, reduced adiposity, and greater cardiovascular fitness [3]. The present data indicate that the ability to increase FAO upon exposure to a physiologically relevant lipid mixture (oleate:palmitate) was significantly dampened in skeletal muscle cells from obese compared to lean subjects, which was not explained by differences in mitochondrial content (Fig. 1B). When measuring mitochondrial respiration in permeabilized cells, we did not find differences in basal or maximal respiration rates, nor for state 3 respiration supported by succinate + rotenone, which tests flux through ETS complex CII (Fig. 1C). These results suggest that lipid metabolic inflexibility in HSkMC from obese humans with respect to lipid oxidation does not involve deficits in ETS complex function. Therefore, differences in the response to 24hFA likely occur either at the tricarboxylic acid or TCA cycle, where the CO_2 is produced, or at upstream events in fatty acid metabolism. Previous reports in skeletal muscle tissue and cells from humans with obesity or type 2 diabetes have also shown normal electron transport function, particularly when data are normalized for mitochondrial content[28,29]. Likewise, we and others have shown deficits in TCA cycle and fatty acid transport/metabolism specifically in humans and animal models of obesity [6,8,30–32].

FENO was used to determine if FAO can be increased in metabolically inflexible HSkMC via PPARα activation [14,33]. Results in animal studies have been mixed, with some findings suggestive of FENO-induced increases in fatty acid oxidation [34–36] and others not [37]. In the present study, we found that short-term exposure to FENO (2h) increased FAO in metabolically inflexible HSkMC, indicating that the effects of FENO are fast-acting and transient. Based on these findings, we hypothesized that the effects of FENO on FAO were independent of PPARα, perhaps acting through a more short-term metabolic activation, such as AMPK phosphorylation, which has been previously reported [38,39]. AMPK is sensitive to the cellular energy state and is a potent activator of FAO, shifting substrate choice to favor lipd over carbohydtates [40]. However, co-incubation of HSkMC with FENO and either the PPARα inhibitor GW6471 or the AMPK inhibitor Compound C eliminated FENO-mediated increases in FAO (Fig. 3A), suggesting that FENO may be acting through *both* PPARα and AMPK. Several studies have reported increased activity of AMPK by FENO [38,39] but the interactions between AMPK and PPARα, and the activation of these

proteins themselves, are complex and often contradictory [41–43], including some evidence of AMPK inhibition of PPARα [43]. In understanding these interactions, the utility of the HSkMC system lies in the ability to incubate human skeletal muscle under conditions which would be difficult to replicate *in vivo*; the finding of FENO functioning through AMPK in terms of modulating FAO thus provides important information relative to the PPARα-AMPK axis.

Due to the potential effectiveness of the intervention, we investigated whether *in vivo* FENO treatment in individuals with pre-diabetes increased FAO in a manner similar to that in the primary muscle cell cultures. While some studies have shown that FENO improves peripheral insulin sensitivity in patients with metabolic syndrome [16,17], our findings confirm many others in terms of there being no effect [14,18–20]. Neither did we observe FENO-induced effects on mRNA content of PPARα-responsive genes or for mitochondrial enzyme activity. However, in a subset of subjects, we observed increases in FAO in skeletal muscle tissue after FENO treatment (Fig. 4D), which appeared to be induced independently of changes in mitochondrial content (as indicated by citrate synthase activity) or the expression of PPARα responsive genes. The discrepancy between PPARα response to FENO in the patient biopsies versus FENO treatment in the primary muscle cell cultures could be the timing of the exposure to FENO as the last dose of FENO was over 24h prior to skeletal muscle sample collection. Based on our results in primary muscle cells, the effects of FENO are relatively short-lived and, therefore, may not be detected a moreprolonged period after administration. However, when isolated mitochondria from these patients were metabolically stimulated to oxidize fatty acids, FAO was increased at the Post-FENO timepoint, suggesting some residual effect or metabolic adaptation to chronic FENO treatment when tested in vivo. Future investigations should aim to clarify the timing of sample collection in relation to FENO dosing, as well as the acute *vs.* chronic effects of FENO treatment in humans.

In summary, the current data suggest that lipid metabolic inflexibility observed in HSkMC from obese subjects appears not to be a function of differences in electron transport flux, but rather to events upstream of electron transport, such as decrements in TCA cycle flux or β-oxidation. Short-term FENO increased FAO through both PPARα and AMPK activation in vitro. Taken together, these novel results suggest that PPARα activators may improve metabolic flexibility acutely, but their role in skeletal muscle metabolism in vivo requires further investigation.

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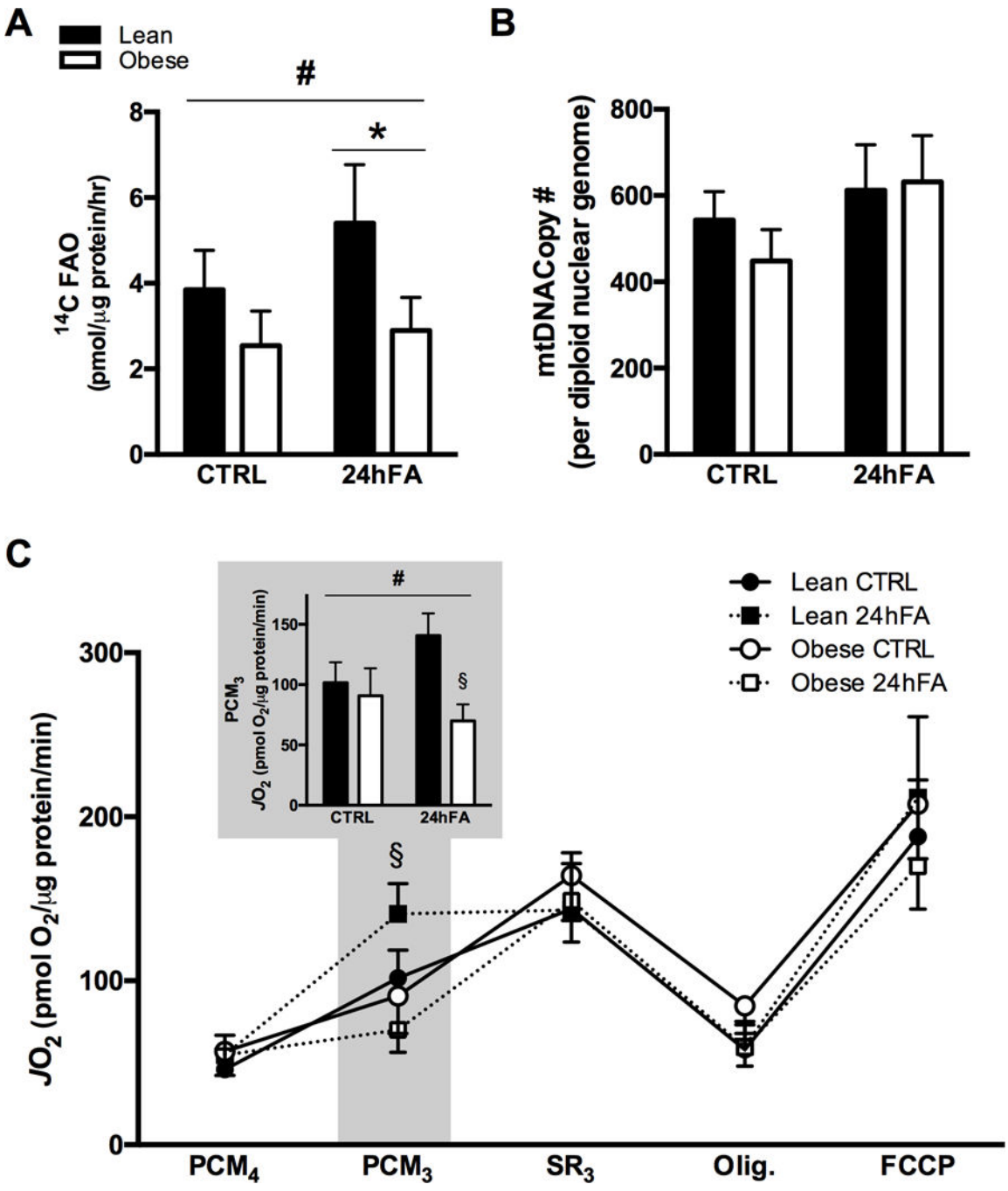

Figure 1.

24hFA exposure increases lipid oxidation in HSkMC from lean but not obese subjects. HSkMC from lean (open bars) and obese (filled bars) were incubated with or without 200 μM fatty acid for 24 h (24hFA) prior to measures of FAO from ^{14}C-labeled palmitate:oleate or mitochondrial respiration of permeabilized HSkMC. Complete FAO was increased with 24hFA treatment in both lean and obese HSkMC, though the response was more robust in lean HSkMC (*A*). There were no differences between groups, nor for 24hFA, in mitochondrial DNA Copy number (*B*). In permeabilized HSkMC, subsequent compound

additions in the Seahorse from basal (PCM_4; palmitoyl carnitine + malate) were ADP (PCM_3), succinate + rotenone (SR_3), oligomycin, and FCCP (*C*). Only PCM_3 showed differences, where a significant ANOVA interaction revealed 24hFA-induced increases in PCM_3 in HSkMC from lean, and PCM_3 decreases in HSkMC from obese subjects (*C*, inset). Data are mean ± SEM. # indicates significant repeated measures ANOVA interaction. * indicates significant effect of 24hFA treatment for both lean and obese, combined. § indicates significant difference from lean in 24hFA condition.

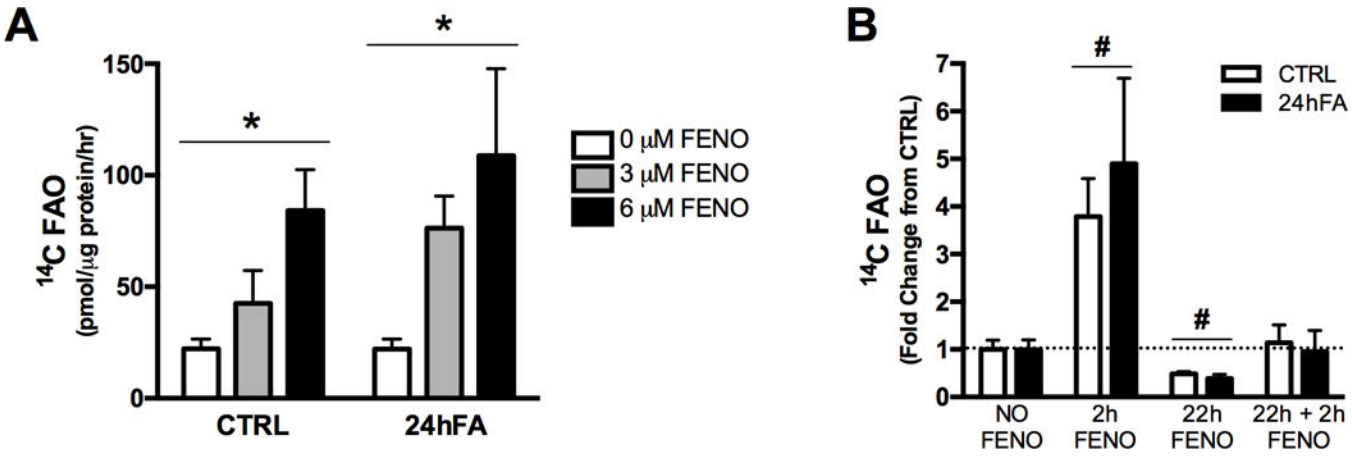

Figure 2.

Acute FENO treatment increases FAO in HSkMC with lipid metabolic inflexibility. Concentration courses of FENO treatment show that 6 μM FENO treatment is most robust for increasing ^{14}C labeled palmitate:oleate FAO in obese, metabolically inflexible HSkMC (*A*). More prolonged treatment with 6 μM FENO for acute (2h), prolonged (22h), or acute + prolonged (2h + 22h) FENO treatments did not show similar robust increase in FAO (*B*). Data represent metabolically inflexible HSkMC from n=2–4 obese subjects, measured in triplicate. Data are mean ± SEM. * indicates significant dose effect for FENO concentration. # indicates significant difference from NO FENO for CTRL and 24hFA combined.

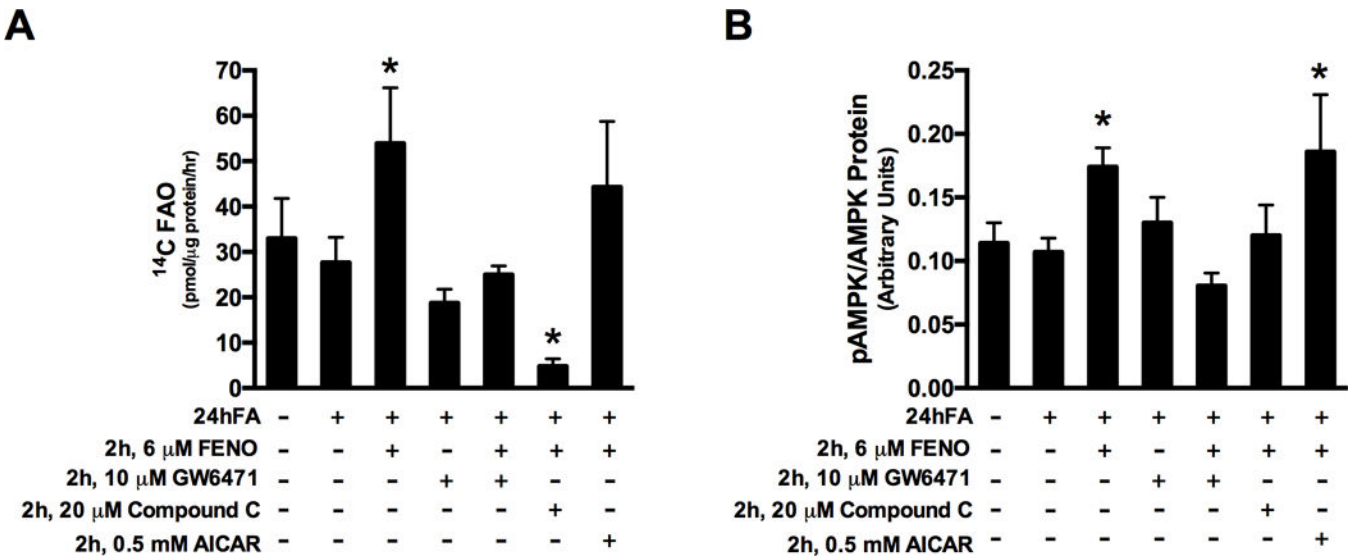

Figure 3.

FENO may act through PPARα and AMPK phosphorylation. Treatment of obese, metabolically inflexible HSkMC with FENO in combination with GW6471, Compound C, or AICAR as indicated, show that FENO-induced increases in FAO are inhibited by co-incubation with PPARα antagonist or AMPK inhibitor, though FENO treatment with AMPK activation by AICAR does not increase FAO beyond that of FENO alone (*A*). Similar observations are made for phosphorylated AMPK/Total AMPK protein levels (Thr172) (*B*). Data are mean ± SEM. * indicates significant difference from 24hFA condition.

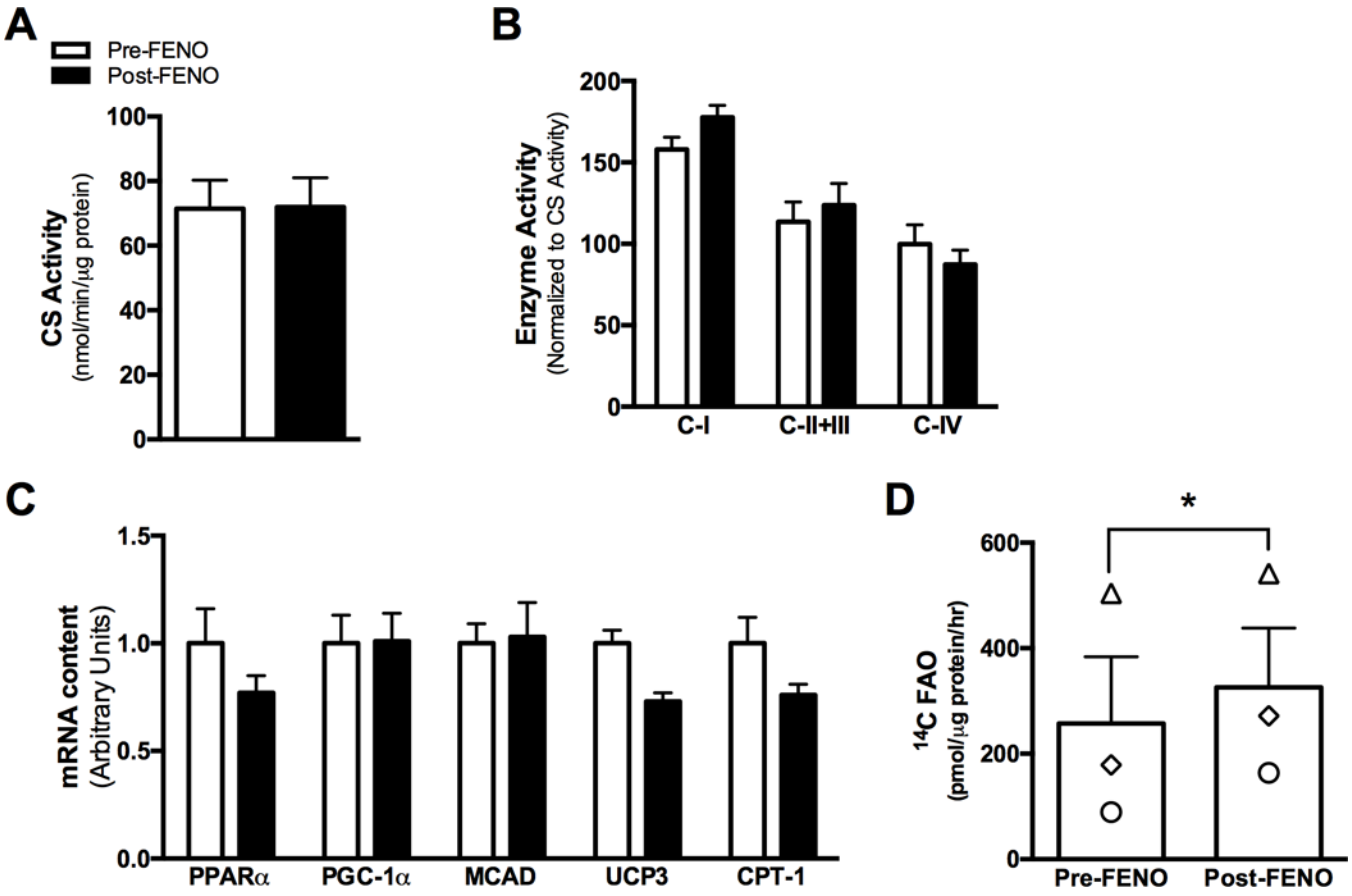

Figure 4.

In vivo FENO treatment does not affect skeletal muscle mitochondrial enzyme activity or genes or proteins linked with PPARα. Skeletal muscle citrate synthase activity (*A*) mitochondrial ETS complex enzyme activities (*B*), mRNA content of genes linked with PPARα activation (*C*) were not different after 10 weeks of FENO treatment, though complete FAO from (^{14}C-labeled palmitate) was increased in fresh skeletal muscle tissue homogenates of a subset of pre-diabetic patients treated with FENO for 10-weeks (*A*). Data are mean ± SEM. * indicates significant difference from Pre-FENO condition.

Table 1.

Patient Characteristics for HSkMC and Skeletal Muscle Tissue Experiments

	HSkMC Subjects		Pre-Diabetic Subjects	
	Lean (n=10)	**Obese (n=10)**	**Pre-FENO (n=11)**	**Post-FENO --**
Age (y)	22.4 ± 0.6	24.2 ± 1.1	48.8 ± 3.0	--
Sex (M/F)	(10/0)	(10/0)	6/5	--
BMI (kg/m^2)	21.6 ± 0.7	36.4 ± 1.7 *	35.9 ± 1.3	37.0 ± 1.5 #
Body Fat (%)	17.8 ± 3.1	41.1 ± 1.8 *	38.1 ± 3.2	38.6 ± 2.7
Glucose (mmol/L)	4.9 ± 0.1	5.0 ± 0.1	5.7 ± 0.3	5.7 ± 0.3
Insulin (μIU/L)	4.6 ± 0.7	16.0 ±1.5 *	12.7 ± 2.8	10.9 ± 2.0
HOMA-IR	1.0 ±0.2	3.5 ± 0.3 *	3.4 ± 1.0	2.9 ± 0.6
Triglycerides (mg/dL)	103.4 ± 14.2	134.6 ± 23.2	161.8 ± 33.5	92.1 ± 11.8 #
Hemoglobin A1c (%)	--	--	5.8 ±0.2	5.9 ± 0.2
S_I (10^{-4}min^{-1}/μU/mL)	--	--	1.5 ± 0.2	1.9 ± 0.3

* indicates significant difference from Lean.

# indicates significant difference from Pre-FENO.