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Effects of acute hyperinsulinemia on skeletal muscle mitochondrial function, reactive oxygen species production, and metabolism in premenopausal women

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Abstract

Background—Acute metabolic demands that promote excessive and/or prolonged reactive oxygen species production may stimulate changes in mitochondrial oxidative capacity.

Purpose—To assess changes in skeletal muscle H₂O₂ production, mitochondrial function, and expression of genes at the mRNA and protein levels regulating energy metabolism and mitochondrial dynamics following a hyperinsulinemic-euglycemic clamp in a cohort of 11 healthy premenopausal women

Methods—Skeletal muscle biopsies of the vastus lateralis were taken at baseline and immediately following the conclusion of a hyperinsulinemic-euglycemic clamp. Mitochondrial production of H₂O₂ was quantified fluorometrically and mitochondrial oxidation supported by pyruvate, malate, and succinate (PMS) or palmitoyl carnitine and malate (PCM) was measured by high-resolution respirometry in permeabilized muscle fiber bundles. mRNA and protein levels were assessed by real time PCR and Western blotting.

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Disclosure

The authors have declared no conflict of interest.

Author Contributions

GF designed the study, participated in data collection, conducted statistical analysis, and drafted the manuscript. JW drafted the manuscript, participated in data collection, and assisted with data analysis. FO assisted with study design and oversaw the clamp experiments. SB participated in data collection. SW conducted the muscle biopsies and assisted with the study design. BG assisted with study design and participated in data collection. All authors made substantial contributions to the intellectual content of the manuscript and have approved the final submitted version.

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Results—H₂O₂ emission increased following the clamp (P<0.05). Coupled respiration (State 3) supported by PMS and the respiratory control ratio (index of mitochondrial coupling) for both PMS and PCM were lower following the clamp (P<0.05). IRS1 mRNA decreased, whereas PGC1 α and GLUT4 mRNA increased following the clamp (P = 0.05). PGC1 α , IRS1, and phosphorylated AKT protein levels were higher after the clamp compared to baseline (P<0.05).

Conclusions—This study demonstrated that acute hyperinsulinemia induced H₂O₂ production and a concurrent decrease in coupling of mitochondrial respiration with ATP production in a cohort of healthy premenopausal women. Future studies should determine if this uncoupling ameliorates peripheral oxidative damage, and if this mechanism is impaired in diseases associated with chronic oxidative stress.

Keywords

hyperinsulinemia; respirometry; mitochondrial plasticity; mitochondrial uncoupling

1. Introduction

The incidence of obesity and associated cardiometabolic diseases, such as insulin resistance (IR) and type 2 diabetes (T2D), continues to rise throughout the westernized world [1]. Skeletal muscle mitochondrial dysfunction and chronic overproduction of reactive oxygen species (ROS) have been considered as primary mediators in their development [2, 3]. In contrast, it has been shown in a rodent model that acute increases in superoxide (O₂^{•-}) and hydrogen peroxide (H₂O₂) from the electron transport system (ETS) may actually have beneficial effects on skeletal muscle insulin sensitivity [4]. Thus, assessing mitochondrial responses to acute metabolic challenges (such as hyperinsulinemia) may be a valuable way to differentiate between normal physiological responses following nutrient load and chronic disturbances (i.e. excessive caloric intake, inactivity, etc.) that have been linked to cardiometabolic diseases.

Mitochondria are known to exhibit a great deal of plasticity in order to respond adequately to chronic stimuli that may alter metabolic demands. For example, exercise training can increase mitochondria content [5] and less than two weeks of exercise training can increase mitochondrial content, enzyme activity, and capacity [6]. Additionally, chronic inactivity has been shown to decrease mitochondria content and oxidative phosphorylation capacity [7], whereas caloric restriction increases substrate oxidation that is linked to ATP production (coupled respiration) and cold stress increases substrate oxidation that is uncoupled from ATP production (uncoupled respiration) [8]. Mitochondrial responses to acute stimuli are less well characterized, but it is thought that mitochondria exhibit a number of adaptations to acute metabolic signals that reflect the present environment. For example, uncoupling mitochondrial respiration from ATP synthesis, by allowing protons to leak across the inner mitochondrial membrane independent of ATP synthase, may be a mechanism to create inefficiency in the presence of abundant substrate and low energetic demand [9, 10]. Uncoupled respiration ensures continued utilization of substrate, promotes the continued movement of electrons through the ETS, and alleviates an elevated proton motive force despite decreased ATP demand [11]. This process is known to be stimulated by ROS production [12] and is thought to be a mechanism to prevent excessive ROS production that

may lead to oxidative damage. However, whether this uncoupling action occurs following an acute metabolic insult known to promote ROS production in humans is unclear. Additionally, the morphology of the dynamic mitochondrial reticular network is altered by acute stimuli, which may also be a function of ROS production [13]. However, morphological properties of mitochondria relative to their function are not well characterized as ROS production has been associated with both fusion [14] and fission [15] processes. A better understanding of the regulation of acute mitochondrial function and dynamics in response to ROS production may yield therapeutic approaches to prevent or mitigate oxidative damage.

The hyperinsulinemic-euglycemic clamp technique has been used to assess acute mitochondrial responses to elevated substrate load [2, 8, 16]. Earlier investigations have shown lower insulin-stimulated ATP production in isolated mitochondria from individuals with T2D compared to controls [16], and lower ATP synthetic rates *in vivo* in patients with T2D [17] and insulin-resistant offspring [2] compared to controls. Thus, insulin-stimulated ATP production appears to be impaired in individuals with impaired insulin sensitivity, however no measure of oxidative phosphorylation efficiency was assessed in these studies. The ability to uncouple mitochondrial respiration from ATP production may be a key response to ROS production that protects against prolonged increases in ROS.

The purpose of the present study was to test the hypothesis that the mitochondria of healthy (premenopausal, non-diabetic) women would display an increase in uncoupled respiration following acute hyperinsulinemia, in conjunction with an increase in ROS production. To test this hypothesis, we examined changes in skeletal muscle H_2O_2 production, mitochondrial bioenergetics using high-resolution respirometry, and expression of genes at the mRNA and protein levels regulating energy metabolism and mitochondrial dynamics in permeabilized fiber bundles from skeletal muscle biopsies obtained before and following a hyperinsulinemic-euglycemic clamp.

2. Materials and Methods

2.1 Participants

Participants were 11 generally healthy premenopausal women. Inclusion criteria were a body mass index (BMI) of 18.5–35 kg/m², sedentary (<30 minutes of structured activity per week), and fasting serum glucose <100 mg/dL. Participants were excluded if they reported use of oral contraceptives, use of any medication known to affect metabolism or glucose tolerance, use of anti-hypertensive agents, history of eating disorder, use of tobacco, change in weight greater than 5 lbs in the previous 6 months, active engagement in unusual dietary practices (e.g. “low-carb” diets), or participation in extreme exercise. All testing was conducted in the first 10 days of the follicular phase of the menstrual cycle. All women provided written informed consent before participating in the study. This study was approved by the Institutional Review Board at the University of Alabama at Birmingham (UAB).

2.2 Study design

Each participant presented after a 12 hr overnight fast. An initial baseline percutaneous needle biopsy of the vastus lateralis was obtained. A portion of tissue was immediately prepared for mitochondrial respirometry and H₂O₂ emission experiments, and remaining tissue was snap frozen in liquid N₂ to be used for mRNA and protein analyses conducted later. Following the baseline biopsy, each participant underwent a hyperinsulinemic-euglycemic clamp lasting approximately 2 hr. A second post-clamp biopsy in the contralateral leg was obtained immediately following the conclusion of the clamp procedure and prepared in the same manner as the baseline biopsy.

2.3 Total and regional body fat quantification

Body composition was assessed during the screening visit by total body scan from dual-energy X-ray absorptiometry (DXA) using a Lunar iDXA densitometer and Core Scan software (GE-Lunar Corporation, Madison, WI). Participants were scanned in light clothing lying supine with arms at their sides. The scans were assessed for total body fat mass, leg fat mass, android fat mass, and intra-abdominal adipose tissue (IAAT) volume.

2.4 Laboratory analyses

The Core Laboratory of the UAB Nutrition Obesity Research Center, Diabetes Research Center, and Center for Clinical and Translational Science analyzed all serum samples. Serum glucose, total cholesterol, HDL cholesterol, triglycerides, and circulating free fatty acids were assessed by SIRRUS analyzer (Stanbio Laboratory, Boerne, TX). Fasting LDL cholesterol was calculated using the method of Friedewald [18]. Serum insulin was measured using a TOSOH AIA-II analyzer (TOSOH Corp., South San Francisco, CA).

2.5 Hyperinsulinemic-euglycemic clamp

A hyperinsulinemic-euglycemic clamp was administered providing a continuous infusion of regular insulin (Humulin, Eli Lilly & Co., Indianapolis, IN) at 40 mU/m² body surface area/min through the brachial vein. Body surface area was calculated using the method of Du Bois [19]. Blood glucose was monitored at bedside at 5 min intervals using a YSI 2300 STAT Plus (YSI Life Sciences, Yellow Springs, OH) and a 20% dextrose solution was infused at a variable rate to maintain euglycemia (targeting 90 mg/dL). Serum samples were collected every 10 minutes for future analysis. The procedure lasted approximately 2 hr. The steady state period was defined for each individual as a period of 30 min or longer (at least 1 hr after beginning the insulin infusion) during which the coefficient of variation for serum glucose, serum insulin, and the recorded dextrose infusion rate was less than 5%. The glucose disposal rate (GDR) was calculated during steady state conditions and is presented as milligrams of dextrose infused per kg of fat-free body mass per minute. The Insulin Sensitivity Index (ISI) is presented as the GDR of each individual normalized to her average steady state serum insulin concentration [20]. Insulin resistance was assessed for each participant using homeostatic model assessment of insulin resistance (HOMA-IR), which was calculated as the following: fasting glucose (mg/dl) × fasting insulin (μU/ml)/405 [21].

2.6 Muscle biopsies

Skeletal muscle samples were obtained at baseline and immediately following the conclusion of the clamp. Samples were obtained from the vastus lateralis under local anesthesia (1% lidocaine) using a 5 mm Bergstrom biopsy needle with suction. Muscle tissue collected at baseline and following the clamp was processed immediately following each biopsy. The tissue was cleaned of adipose and connective tissue. A bundle of approximately 20 mg was selected for mitochondrial experiments and transported in Buffer X (50 mM K-MES, 7.23 mM K₂EGTA, 2.77 mM CaK₂EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.7 mM ATP, 14.3 mM phosphocreatine, and 6.56 mM MgCl₂ (pH 7.1, 290 mOsm)) on ice [22]. The remaining tissue was frozen immediately in liquid N₂ and subsequently stored at -80°C mRNA and protein quantification.

2.7 Preparation of permeabilized muscle fiber bundles

This technique has been adapted from previously published methods [22]. The biopsied tissue was dissected into several smaller muscle bundles (of approximately 1.0–5.0 mg wet weight). Each bundle was gently separated longitudinally with a pair of antimagnetic needle-tipped forceps under magnification in Buffer X. Bundles were weighed and then treated with 30 µg/ml saponin in Buffer X on a rotator for 30 min at 4°C. Next, the tissue bundles were washed for 15 min in Buffer Z (105mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K₂HPO₄, 5 mM MgCl₂, 5 µM glutamate, 2 µM malate, and 5.0 mg/ml BSA (pH 7.4, 290 mOsm)). Finally, the samples were transferred to Buffer Z supplemented with 5 µM blebbistatin and 20 mM creatine hydrate for 10 minutes prior to experiments. Blebbistatin was present during all respirometry and H₂O₂ experiments to prevent contraction of the myofibers [22].

2.8 H₂O₂ quantification

Using a permeabilized fiber bundle of approximately 1.0–1.5 mg, H₂O₂ emission was measured fluorometrically at 37°C in Buffer Z containing 5 µM blebbistatin, 20 mM creatine hydrate, 10 µM Amplex Ultra Red, and 3 U/mL horseradish peroxidase. Oxidation of Amplex Ultra Red to resorufin was monitored using a Fluoromate SF-2 spectrofluorometer (SCINCO, Seoul, South Korea) with temperature control and magnetic stirring at more than 1,000 rpm detecting at excitation/emission λ = 568/581 nm. For each experiment, resorufin fluorescence was converted to pmol H₂O₂ via an H₂O₂ standard curve generated under identical substrate conditions with the exception of the permeabilized fiber bundles. H₂O₂ emission during coupled respiration (State 3) was measured after the addition of 9 mM pyruvate, 4 mM malate, 2.5 mM succinate and 1 mM ADP. H₂O₂ emission during uncoupled respiration (State 4) was measured after the addition of 2 µg/mL oligomycin. H₂O₂ emission was normalized to the wet weight of the fiber bundle taken prior to each experiment.

2.9 Mitochondrial respiration

High-resolution respirometry experiments were performed using an Oxygraph O2K (Oroboros Instruments, Innsbruck, Austria) containing 2 mL of Buffer Z with 5 µM blebbistatin and 20 mM creatine hydrate, constantly stirred at 37°C under conditions of O₂

saturation, containing a prepared permeabilized fiber bundle of approximately 3.0–5.0 mg. Two substrate protocols were used: 9 mM pyruvate, 4 mM malate, and 2.5 mM succinate (PMS) to drive convergent electron input to complexes I and II of the ETS or 40 μ M palmitoyl carnitine and 2 mM malate (PCM) to examine mitochondrial fatty acid oxidation. State 3 in each substrate condition was measured after the addition of 1 mM ADP. Cytochrome *c* (10 μ M) was added to assess the mitochondrial membrane integrity. There was no significant difference in State 3 respiration assessed after cytochrome *c* addition. State 4 was induced by the addition of 2 μ g/mL oligomycin. Oxygen flux was normalized to the wet weight of each fiber bundle taken prior to experiments [23]. The respiratory control ratio (RCR) was calculated as State 3/State 4, providing a measure of mitochondrial coupling.

2.10 Quantitative PCR

Muscle samples of approximately 30 mg were powdered in a liquid nitrogen-cooled mortar and pestle and homogenized in Trizol Reagent (Life Technologies; Carlsbad, CA) following manufacturer's procedure to obtain mRNA. mRNA concentration was determined using a NanoDrop Lite (Thermo Scientific, Wilmington, DE). mRNA quality was determined using the A_{260}/A_{280} ratio. iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA) was used to convert mRNA to double-stranded cDNA. Quantitative real-time PCR was performed using SsoAdvanced SYBR Green 2x Supermix (Bio-Rad Laboratories) in a 20 μ L reaction using a CFX Connect RT-PCR Detection System (Applied Biosystems, Foster City, CA), following manufacturer's instructions. All samples were analyzed in triplicate. Relative quantification values ($-CT$) were calculated for each target gene relative to Human GAPDH Endogenous Control (Applied Biosystems), which was run on each individual plate. CT values were used for correlation analyses with other study variables. Results depicted are shown as the mean and range of relative fold differences compared to baseline [24]. Expression was assessed using the following primers (Applied Biosystems by Life Technologies, Warrington UK): glucose transporter type 4 (GLUT4) (Hs 00168966 m1), AKT substrate of 160 kDa (AS160) (Hs 00207999 m1), Protein kinase B (AKT1) (Hs 00178289 m1), insulin receptor substrate 1 (IRS1) (Hs 00178563 m1), pyruvate dehydrogenase kinase 4 (PDK4) (Hs 01037712 m1), uncoupling protein 3 (UCP3) (Hs 01106052 m1), fatty acid translocase (CD36) (Hs 01567185 m1), carnitine palmitoyltransferase 1B (CPT1B) (Hs 03046298 s1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) (Ms 01016719 m1), sarcolipin (SLN) (Hs 00161903 m1), Cu-Zn superoxide dismutase (SOD1) (Hs 00533490 m1), and Mn superoxide dismutase (SOD2) (Hs 00167309 m1).

2.11 Immunoblotting

Muscle samples of approximately 30 mg were powdered in a liquid N₂-cooled mortar and pestle and homogenized in 6 μ L/mg muscle of ice-cold lysis buffer (150mM NaCl, 50 mM Tris-HCl, 5mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 0.5% NP-40 at pH 7.4) with protease and phosphatase inhibitors. Each sample was centrifuged at 15,000 g for 15 min at 4°C and the supernatant was collected. Protein content of the supernatant was quantified using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Thirty-seven micrograms of protein were treated with 4x NuPAGE LDS Sample Buffer (Novex,

Carlsbad, CA) and 10x NuPAGE Reducing Agent (Novex) and incubated at 70°C for 10 min. The samples were electrophoresed in an SDS polyacrylamide gel (4–20%) at 100 V in an ice bath. The gels were blotted to polyvinylidene fluoride membranes using a semi-dry transfer method at 25 V for 12 min using a Pierce Power Blotter (Thermo Fisher Scientific). The membranes were blocked under conditions optimized for each antibody (2–5% milk and/or 2–5% BSA in 1 × PBS with 0.1% Tween 20 (PBST)) for 1 hr at room temperature with gentle agitation. Primary antibody was added at an appropriate dilution for incubation overnight at 4°C with gentle agitation. We probed for the following: rabbit polyclonal against total acetyl-CoA carboxylase (ACC) (1:1000, Cell Signaling Technology, #3662), P-ACC (1:500, Cell Signaling Technology, #3661), P-AKT (Ser473) (1:1000, Cell Signaling Technology, #9271), P-AS160 (1:1000, Cell Signaling Technology, #4288), CD36 (1:1000, Santa Cruz Biotechnology, sc-9154), GLUT4 (1:2000, Abcam, ab654), IRS1 (1:1000, Abcam, ab66153), and UCP3 (1:500, Abcam, ab3477); mouse monoclonal against dynamin-related protein 1 (DRP1) (1:500, Abcam, ab56788), Mitofusin 1 (MFN1) (1:1000, Abcam, ab57602), and Mitofusin 2 (MFN2) (1:2000, Abcam, ab56889); rabbit monoclonal against α -tubulin (1:1000, Cell Signaling, #2125), CPT1B (1:1000, Abcam, ab134135), and glycogen synthase 1 (GS1) (1:10000, Abcam, ab40810); and a goat polyclonal against PGC1 α (1:1000, Abcam, ab106814) and lipoprotein lipase (LPL) (1:1000, R&D Systems, AF7197). Horseradish peroxidase-conjugated secondary antibodies were used at 1:50,000 in 0.5% of selected blocking agent in PBST for 1 hr at RT with gentle agitation. Bands were visualized by chemiluminescent detection using ECL Western Blotting Substrate (Pierce Biotechnology) in a ChemicDoc XRS (Bio-Rad Laboratories). Band densitometry was quantified by Image Lab software (Version 4.1) (Bio-Rad Laboratories). Values shown were obtained after normalization to the reference protein α -tubulin. P-ACC was further normalized to total ACC density.

2.12 Statistical analyses

All statistical analyses were conducted using SPSS Statistics for Macintosh Version 22.0 (IBM Corp., Armonk, NY). Descriptive characteristics are reported as mean $\pm$ standard deviation and outcome variables are depicted as mean $\pm$ standard error of the mean. Differences for dependent variables between baseline and post-clamp were assessed using the Wilcoxon signed-rank test. Relationships between variables were determined using Spearman rank-order correlations. An alpha level of 0.05 was used to determine statistical significance.

3. Results

3.1 Participant characteristics

Baseline demographic, body composition, and biochemical data are presented in Table 1. One participant had a fasting glucose of 114 mg/dL indicating impaired fasting glucose, however she displayed no other outlying biochemical, body composition, or mitochondrial outcome measures. Analyses were performed including and excluding this participant. There were no differences in statistical outcomes for any variables; therefore we included her data in the final analyses. Body composition data and GDR (normalized to FFM) are limited to 10 participants, as one participant did not complete her DEXA.

3.2 H₂O₂ production and mitochondrial respirometry

We first assessed skeletal muscle ROS production by quantifying H₂O₂ production in permeabilized fiber bundles from samples obtained at baseline and post-clamp. Compared to baseline values, post-clamp State 3 H₂O₂ emission increased by 59% and post-clamp State 4 H₂O₂ emission increased by 57% using PMS ($P<0.05$) (Figure 1A).

Following the clamp procedure, State 3 respiration decreased by 30% ($P<0.01$) with no significant change during State 4 using PMS (Figure 1B), demonstrating a decrease in oxidative phosphorylation capacity. There were no significant changes in mitochondrial respiration under PCM (Figure 1C). The RCR decreased by 37% using PMS and 31% using PCM following the clamp ($P<0.05$) (Figure 1D), which suggests greater relative mitochondrial uncoupling.

There were no associations between any measures of mitochondrial respiration and H₂O₂ production (data not shown). We further examined a number of correlations between participant characteristics and mitochondrial respiratory and H₂O₂ experiments. BMI and Total Fat Mass exhibited significant negative correlations with pre-clamp State 3 ($r=-0.865$, $P=0.001$ and $r=-0.638$, $P=0.047$, respectively) and State 4 ($r=-0.734$, $P=0.010$ and $r=-0.679$, $P=0.031$, respectively) oxygen consumption under the PMS condition. Additionally, BMI alone was associated with post-clamp State 3 ($r=-0.834$, $P=0.001$) and State 4 ($r=0.657$, $P=0.013$) oxygen consumption under the PMS substrate condition. We found no associations between participant characteristics and any measure of H₂O₂ production.

3.3 mRNA and protein quantification

Skeletal muscle mRNA levels of target genes associated with energy metabolism and mitochondrial morphology are shown in Figure 2. Skeletal muscle IRS1 mRNA was lower following the clamp, whereas there was an increase in PGC1 α mRNA levels ($P<0.05$). Additionally, there was a significant increase in skeletal muscle GLUT4 mRNA ($P=0.05$). There were no significant changes for the remaining target genes. There were a number of significant associations between H₂O₂ emission and mRNA levels (Table 2). There was a significant negative correlation between baseline and post-clamp UCP3 mRNA levels and baseline State 3 and State 4 H₂O₂ emission ($P<0.05$). Baseline and post-clamp UCP3 mRNA levels were negatively associated with baseline State 3 and State 4 H₂O₂ emission ($P<0.05$). Furthermore, baseline SOD1 mRNA levels were negatively correlated with baseline State 3 and State 4 H₂O₂ emission ($P<0.05$). Only baseline SOD2 mRNA levels were negatively correlated with baseline State 3 and State 4 H₂O₂ emission ($P<0.05$).

Skeletal muscle quantity of target proteins is shown in Figures 3–5. Following the clamp, we observed an increase in the following proteins: IRS1 by 53.9% (Figure 3B), phosphorylated AKT by 24.6% (Figure 3E), and PGC1 α by 25.6% (Figure 5E) ($P<0.05$). There were no other significant changes for the remaining target proteins. We observed several significant correlations between H₂O₂ emission and proteins related to mitochondrial fusion (Table 3). Baseline MFN2 protein density was positively associated with baseline State 3 and State 4 H₂O₂ emission ($P<0.05$). Post-clamp MFN1 protein density was positively associated with post-clamp State 3 and State 4 H₂O₂ emission ($P=0.05$). Although they did not reach the

level of significance, the relationship between MFN2 protein density and post-clamp State 3 and State 4 H₂O₂ emission displayed a similar trend (P<0.1). No associations were found between H₂O₂ emission and DRP1 protein density.

4. Discussion

Shifts in energy balance can influence the cellular redox environment in favor of greater oxidative stress [25]. This relationship has been observed chronically following changes in habitual dietary intake [25] and acutely following a single meal [26]. However, whether acute changes in ROS production occur concurrently with the uncoupling of mitochondrial respiration from the production of ATP had not previously been shown. The purpose of this study was to identify potential factors within skeletal muscle that respond acutely to regulate fuel metabolism and the redox environment. The primary finding in this study was that there was an increase in the production of H₂O₂ and an increase in mitochondrial uncoupling in skeletal muscle following a hyperinsulinemic-euglycemic clamp, suggesting a possible link between these processes. Furthermore, we found changes in the expression of genes at the mRNA and protein levels that are intricately involved in insulin signaling and in mediating mitochondrial biogenesis and dynamics. We also found that mRNA levels of genes associated with uncoupling mitochondrial oxidative phosphorylation and antioxidant enzymes were inversely related to H₂O₂ production. Additionally, key proteins known to promote mitochondrial fusion processes were related to higher skeletal muscle H₂O₂ production. These observations support the hypothesis that the ability of the mitochondria to adapt readily to a metabolic demand, specifically by altering efficiency, may be an important mechanism induced by ROS production in healthy individuals for the purpose of regulating the ROS production.

Our data contrast with a previous investigation that found an increase in mitochondrial efficiency using rodent and human myoblasts treated with insulin for 20 minutes [27]. There are a number of differences between the approaches taken in these studies, including the tissues examined and length of the insulin treatment, which may explain the discrepant findings. However, when taken together, data from these two studies may offer further insight into the effect of insulin and glucose on mitochondrial function. The duration of the clamp procedure is approximately two hours, thus we speculate that the present study has characterized mitochondrial function when energy reserves within the muscle have become saturated, electron transport has slowed, and ROS production has increased to promote an uncoupling response in the mitochondria. We hypothesize that our findings would corroborate those of Nisr and colleagues (higher coupling efficiency following 20 minutes of insulin stimulation) if an additional biopsy sample had been obtained during the early phases of the clamp procedure, as restoring ATP concentrations following a fasting period would be a priority.

Prior investigations have shown that acute hyperinsulinemia can increase ROS production [28]. Additionally, postprandial hyperglycemia can promote the production of ROS and oxidative stress especially when carbohydrate and lipid sources are consumed in abundance [29, 30]. The results from this study confirm these findings as we have demonstrated an increase in skeletal muscle H₂O₂ production of greater than 50% following the clamp. Thus,

an increase in ROS in the postprandial state over several months or years may lead to chronic oxidative stress and exacerbate pathophysiological processes associated with the onset of cardiometabolic diseases. It is important to mention that some level of ROS is necessary to activate redox-signaling pathways that are known to have a role in a number of homeostatic cellular processes, including insulin signaling [31]. However, when ROS are produced in excess of the antioxidative capacity of the cell, these molecules may oxidize nearby lipids, proteins, nucleic acids, and enzymes, altering the function of these cellular components [32]. Thus, the ability of the mitochondria to regulate ROS production combined with antioxidant defense mechanisms is imperative to maintaining homeostatic redox environment and preserving cardiometabolic health.

Uncoupling electron transport and proton re-entry from ATP synthesis is thought to limit ROS production and in fact seems to be stimulated by ROS [33]. It is possible that increased uncoupled respiration following the clamp observed in this study is an adaptive mechanism to reduce acute ROS production during the postprandial state and thus prevent chronic oxidative stress. Therapeutic compounds (e.g. 2,4-dinitrophenol (DNP)) that alter mitochondrial efficiency have been investigated as a means to combat obesity, cardiometabolic diseases, and fatty liver disease [34]. However, to date these compounds have yielded equivocal results. In humans, DNP in particular promotes weight loss [35], while others have shown in rodents that uncoupling agents targeting hepatic mitochondria can resolve hypertriglyceridemia and IR [36]. However, use in humans has been limited due to numerous deleterious side effects (e.g. tachycardia, hyperthermia) [35]. These symptoms are primarily due to an increase in chronic and systemic mitochondrial uncoupling. The present study supports the need for future studies to identify uncoupling agents and/or lifestyle modifications that can increase mitochondrial uncoupling in a timed and targeted manner as a possible means to attenuate ROS production during shifts in energy balance in contrast to chronic and indiscriminate uncoupling agents [37].

Changes at the molecular level provided additional evidence for a shift in processes that regulate energy metabolism following the clamp. Although IRS1 mRNA decreased following the clamp, IRS1 protein increased. Similarly, decreases in IRS1 mRNA following hyperinsulinemia have been demonstrated in several previous studies [38, 39]. It is possible that increased translation of mRNA to protein during hyperinsulinemia is responsible for the imbalance between mRNA and protein changes. We also observed an increase in both the gene transcript and protein levels of PGC1 α . PGC1 α mRNA increases in response to acute metabolic stressors [40] and has been shown in a number of studies to regulate mechanisms that mediate mitochondrial dynamics [41, 42].

Mitochondrial dynamics, the balance between fusion and fission events, are thought to promote the preservation of mitochondrial health [43] and these processes appear to be regulated by transient metabolic demands. However, we saw no change in mitochondrial fission and fusion proteins (MFN1, MF2, DRP1) in this study. It is possible that changes in these proteins may not occur within the timeframe of our sampling period or they may be chronically regulated. Indeed, a previous study using an exercise model has shown that increases in PGC1 α were observed 2 hr post-exercise, but that MFN1 and MFN2 were only significantly elevated after 24 hr [44]. Despite not observing any changes in MFN1 and

MFN2, these proteins did exhibit associations with H₂O₂ production, such that higher MFN1 (post-clamp) and MFN2 (baseline) were associated with greater H₂O₂ emission. Previous investigations do not appear to agree on whether an environment favoring fission or fusion is more 'protective' against ROS production and oxidative damage. Some have found increased mitochondrial fusion in response to mitochondrial ROS production [14] (likely in order to reduce ROS, which is supported by the positive associations presented in this study between MFN1/MFN2 and H₂O₂ production) and/or hyperglycemia [45]. However, others consider ROS production to be associated with a pro-fission environment [15] and when fission was inhibited in a transgenic mouse model, uncoupled respiration increased to prevent oxidative stress under hyperglycemic conditions [46]. While only correlative, our data support the idea that increased fusion may be linked to increased mitochondrial ROS production. Finally, protein levels of UCP3, SOD1 and SOD2 assessed at baseline and UCP3 and SOD1 following the clamp were inversely associated with H₂O₂ emission at baseline. UCP3 is a critically important facilitator of uncoupling action in skeletal muscle [47]. Our data suggest that a greater capacity for uncoupling respiration from ATP production contributes to an overall lower fasted level of ROS production. SOD1 is responsible for the production of H₂O₂; thus the observed inverse relationship may reflect an enhanced antioxidative capacity within the intermembrane and cytosolic spaces. Cross-sectional data must be interpreted with caution, as they do not capture dynamic changes that may lend insight into cause and effect.

T2D is associated with reduced mitochondrial capacity, plasticity, and content [48] and greater ROS production [49]. Furthermore, a previous study by Bloomer et al. has shown that a meal challenge can increase markers of oxidative stress in healthy and obese women similarly, but that the obese cohort exhibited elevations in these markers for a longer period following the meal [26]. This may be a function of a reduced oxidative capacity as BMI increases, observed in the present study as a negative association between body fat and mitochondrial oxidative capacity as well as the work of others [50, 51]. Thus, we hypothesize that if the present study design were to be employed in obese participants with obesity and cardiometabolic diseases such as T2D, these participants may exhibit less dramatic or no uncoupling and greater overall ROS production (contributing to oxidative stress) following hyperinsulinemia compared to healthy counterparts. If the mitochondria from individuals with metabolic disease do not exhibit the plasticity necessary to prevent excessive ROS production, this could contribute to a chronic perturbation of the redox environment and disease progression. Future studies should evaluate mitochondrial bioenergetics and ROS production in individuals that present with obesity and cardiometabolic diseases.

Strengths of this study include the use of the hyperinsulinemic-euglycemic clamp as a well-controlled model of the hyperinsulinemic state in contrast to a mixed meal in which the digestion, absorption, and metabolism of macronutrients may have contributed to greater individual tissue variability. Mitochondrial respiration was assessed using high-resolution respirometry in permeabilized fiber bundles, a technique that may more closely mimic an *in vivo* state in contrast to experiments performed using isolated mitochondria. We also acknowledge there are limitations to the present study design. Our conclusions are limited to a study population of healthy premenopausal women, and results may differ in older women,

men, or individuals with cardiometabolic diseases. Additional pre- and post-transcriptional changes, namely those related to mitochondrial dynamics, may have been observed if muscle samples had been obtained at different time points following to the clamp (e.g. 2 hr – 24 hr post-clamp). Last, non-parametric statistical analysis were utilized due to the small sample size, thus limiting our ability to adjust for potential confounding variables.

5. Conclusions

This study demonstrated that acute hyperinsulinemia induced H₂O₂ production and a concurrent decrease in coupling of mitochondrial respiration with ATP production in a cohort of healthy premenopausal women. We believe this decrease in coupling contributes to the attenuation of ROS production in healthy individuals, preventing excess ROS that may contribute to oxidative damage in the postprandial state. Future research is needed to identify if uncoupled mitochondrial respiration can mediate the peripheral oxidative state and if this mechanism is impaired in diseases associated with chronic oxidative stress, such as T2D.

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Abbreviations

ACC	acetyl-CoA carboxylase
AKT	protein kinase B
AS160	AKT substrate of 160 kDa
BMI	body mass index
CD36	fatty acid translocase
CPT1B	carnitine palmitoyltransferase 1B
DNP	2,4-dinitrophenol
DRP1	Dynamin-related protein 1
DXA	dual-energy X-ray absorptiometry
ETS	electron transport system
GDR	glucose disposal rate

GLUT4	glucose transporter type 4
GS1	glycogen synthase 1
HOMA-IR	homeostatic model assessment of insulin resistance
IAAT	intra-abdominal adipose tissue
IR	insulin resistance
IRS1	insulin receptor substrate 1
ISI	insulin sensitivity index
LPL	lipoprotein lipase
MFN1	mitofusin 1
MFN2	mitofusin 2
PCM	palmitoyl carnitine and malate substrate condition
PDK4	pyruvate dehydrogenase kinase 4
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PMS	pyruvate, malate, and succinate substrate condition
RCR	respiratory control ratio
ROS	reactive oxygen species
SLN	sarcolipin
SOD1	Cu-Zn superoxide dismutase
SOD2	Mn superoxide dismutase
T2D	type 2 diabetes
UCP3	uncoupling protein 3

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Figure 1. Changes in skeletal muscle mitochondrial H₂O₂ production and respiration from baseline to post-clamp

Values represent mean $\pm$ SEM. *P 0.05. (A) H₂O₂ emission during State 3 and State 4 respiration supported by pyruvate, malate, and succinate (PMS); (B) State 3 and State 4 respiration supported by PMS; (C) State 3 and State 4 respiration supported by malate and palmitoyl carnitine (PCM); (D) Respiratory control ratio (RCR) for PMS and PCM.

Figure 2. Changes in expression of genes related to energy metabolism and mitochondrial morphology

Mean fold change (relative to baseline values depicted by dashed line) in skeletal muscle mRNA levels from baseline to post-clamp are presented as 2^{-CT} . Error bars represent the possible range of fold differences. *P < 0.05.

Figure 3. Muscle content of proteins related to glucose metabolism and insulin signaling
 Baseline and post-clamp contents were measured were measured using Western blotting normalized to α -tubulin. Values represent mean $\pm$ SEM. *P 0.05. (A) GLUT4, n=11; (B) IRS1, n=11; (C) P-AS160, n=10; (D) E) GS1, n=11; (E) P-AKT, n=10.

Figure 4. Muscle content of proteins related to fatty acid transport and metabolism
Baseline and post-clamp contents were measured were measured using Western blotting with α -tubulin as a loading control. Values represent mean $\pm$ SEM. *P 0.05. (A) CPT1B, n=10; (B) CD36, n=10; (C) LPL, n=10; and (D) P-ACC, n=11.

Figure 5. Muscle content of proteins related to mitochondrial morphology and function
 Baseline and post-clamp contents were measured using Western blotting normalized to α -tubulin. Values represent mean $\pm$ SEM. *P 0.05. (A) DRP1, n=10; (B) MFN1, n=10; (C) MFN2, n=10; (D) UCP3, n=11; (E) PGC1 α , n=11.

Table 1

Participant characteristics (n=11, Mean±SD).

Age (years)	24.7 ± 4.4
Race	7 EA/4 AA
BMI (kg/m ²)	25.3 ± 4.5
Total Fat Mass (kg) [‡]	23.2 ± 8.7
Leg Fat Mass (kg) [‡]	9.1 ± 3.2
Trunk Fat Mass (kg) [‡]	10.8 ± 5.1
Fat Free Mass (kg) [‡]	42.3 ± 4.7
IAAT volume (cm ³) [‡]	261.6 ± 294.0
Total Cholesterol (mg/dL)	168.9 ± 23.4
Triglycerides (mg/dL)	80.9 ± 54.9
HDL (mg/dL)	59.1 ± 13.0
LDL (mg/dL)	93.2 ± 13.0
Free Fatty Acids (mEq/L)	0.7 ± 0.3
Fasting Glucose (mg/dL)	87.9 ± 11.7
Fasting Insulin (μIU/mL)	9.1 ± 4.1
HOMA-IR	2.0 ± 1.3
Insulin Infusion Rate	2.0 ± 1.4
GDR (mg/kgFFM/min) [‡]	13.7 ± 7.3
ISI	15.4 ± 8.7

[‡]n=10.

AA, African-American; BMI, body mass index; EA, European-American; GDR, glucose disposal rate; HOMA-IR, homeostatic model assessment of insulin resistance; IAAT, intra-abdominal adipose tissue; ISI, Insulin Sensitivity Index.

Table 2
Spearman correlations between H2O2 production data and mRNA levels. Correlation coefficient, P-value.

	Baseline mRNA			Post-Clamp mRNA		
	UCP3	SOD1	SOD2	UCP3	SOD1	SOD2
Baseline H2O2	State 3 -0.688, 0.019 *	-0.827, 0.002 *	-0.664, 0.026 *	-0.691, 0.019 *	-0.609, 0.047 *	0.064, 0.853
	State 4 -0.688, 0.019 *	-0.827, 0.002 *	-0.664, 0.026 *	-0.691, 0.019 *	-0.609, 0.047 *	0.064, 0.853
Post-Clamp H2O2	State 3 -0.340, 0.336	-0.442, 0.200	-0.370, 0.293	-0.442, 0.200	-0.139, 0.701	-0.152, 0.676
	State 4 -0.322, 0.364	-0.455, 0.187	-0.406, 0.244	-0.430, 0.214	-0.127, 0.726	-0.115, 0.751

* P<0.05.

Table 3
Spearman correlations between H2O2 production data and protein density. Correlation coefficient, P-value.

	Baseline Protein Density				Post-Clamp Protein Density			
	MFN1	MFN2	DRP1		MFN1	MFN2	DRP1	
Baseline H2O2	State 3	0.418, 0.229	0.673, 0.033 *	-0.336, 0.312	0.430, 0.214	0.091, 0.803	-0.176, 0.627	
	State 4	0.418, 0.229	0.673, 0.033 *	-0.336, 0.312	0.430, 0.214	0.091, 0.803	-0.176, 0.627	
Post-Clamp H2O2	State 3	0.350, 0.356	0.633, 0.067	0.176, 0.627	0.683, 0.042 *	0.633, 0.067	0.450, 0.224	
	State 4	0.350, 0.356	0.633, 0.067	0.236, 0.511	0.683, 0.042 *	0.633, 0.067	0.450, 0.224	

* P<0.05.