Insulin rapidly increases skeletal muscle mitochondrial ADP sensitivity in the absence of a high lipid environment

Henver S. Brunetta1,2*, Heather L. Petrick1*, Bayley Vachon1, Everson A. Nunes2, Graham P. Holloway1

1 Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Canada; 2 Department of Physiological Sciences, Federal University of Santa Catarina, Florianopolis, Brazil

* These authors contributed equally to this work.

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Corresponding author:
Henver S. Brunetta, henver@unicamp.br
Graham P. Holloway, ghollowa@uoguelph.ca
Human Health and Nutritional Sciences,
University of Guelph
491 Gordon Street, Guelph, ON, N1G 2W1, Canada

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Abstract: Reductions in mitochondrial function have been proposed to cause insulin resistance, however the possibility that impairments in insulin signaling negatively affects mitochondrial bioenergetics has received little attention. Therefore, we tested the hypothesis that insulin could rapidly improve mitochondrial ADP sensitivity, a key process linked to oxidative phosphorylation and redox balance, and if this phenomenon would be lost following high-fat diet (HFD)-induced insulin resistance. Insulin acutely (60 minutes post I.P.) increased submaximal (100-1000 μM ADP) mitochondrial respiration ~2-fold without altering maximal (>1000 μM ADP) respiration, suggesting insulin rapidly improves mitochondrial bioenergetics. The consumption of HFD impaired submaximal ADP-supported respiration ~50%, however, despite the induction of insulin resistance, the ability of acute insulin to stimulate ADP sensitivity and increase submaximal respiration persisted. While these data suggest that insulin mitigates HFD-induced impairments in mitochondrial bioenergetics, the presence of a high intracellular lipid environment reflective of an HFD (i.e. presence of palmitoyl-CoA) completely prevented the beneficial effects of insulin. Altogether, these data show that while insulin rapidly stimulates mitochondrial bioenergetics through an improvement in ADP sensitivity, this phenomenon is possibly lost following HFD due to the presence of intracellular lipids.

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Keywords: insulin resistance, mitochondrial function, metabolism, ADP sensitivity, skeletal muscle.

Abbreviations: ADP – adenosine diphosphate; ATP – adenosine triphosphate; H2O2 – hydrogen peroxide; HFD – high-fat diet; HK – hexokinase; NAD – nicotinamide adenine...
dinucleotide; ROS – reactive oxygen species; RCR – respiratory control ratio; RER – respiratory exchange ratio; P-CoA – palmitoyl-CoA.

INTRODUCTION

Skeletal muscle plays a fundamental role in the maintenance of glucose homeostasis, and as a result, a reduction in skeletal muscle insulin responsiveness is implicated in the development of type 2 diabetes mellitus (T2DM). While the underlying mechanisms remain debatable, mitochondrial respiratory dysfunction has been repeatedly linked to intramyocellular lipid accumulation and greater mitochondrial reactive oxygen species (ROS) production, mechanisms intricately linked to the negative effects of overnutrition on insulin signaling (1–6). Interestingly, it has been shown that mitochondrial oxidative phosphorylation might be modulated by insulin, however, it remains unknown if alterations in mitochondrial bioenergetics precede the development of skeletal muscle insulin resistance or occur secondary to attenuations in insulin signaling.

Insulin is a potent anabolic hormone that regulates several metabolic ATP-dependent processes (7). As a result, the absence of insulin action (i.e. insulin deficiency, Akt2 ablation) causes reductions in mitochondrial content, oxidative capacity and ATP concentration while also increasing ROS production (8–10). Moreover, insulin stimulates mitochondrial NADH activity, improves mitochondrial coupling, and upregulates ATP synthesis in cell culture, mice, and humans (11–15), providing compelling evidence that insulin influences mitochondrial bioenergetics. Notably, the effects of insulin on mitochondrial function are partially lost after high fat- or palmitate-induced insulin resistance (11,16), which suggests a bidirectional interaction between reduced insulin sensitivity and abnormalities in mitochondrial function. Therefore, while it has been proposed that chronic insulin stimulates ATP synthesis and increases oxidative
phosphorylation, there is a lack of experimental evidence focusing on the rapid effects of insulin on mitochondrial function, or the influence of insulin resistance in these processes.

Therefore, the present study aimed to investigate the acute effects of insulin on mitochondrial bioenergetics, ADP sensitivity, and ROS emission in skeletal muscle from control and high-fat diet (HFD)-induced insulin resistant mice. We tested the hypothesis that insulin could rapidly improve mitochondrial ADP sensitivity, a dynamic phenomenon that is acutely modulated in several physiological contexts (1,16,17). In addition, we hypothesized the effect of insulin on mitochondrial function would be lost following HFD-induced insulin resistance. Our results suggest that insulin acutely improves mitochondrial ADP sensitivity regardless of the diet consumed. However, the effects of insulin on mitochondrial bioenergetics are mitigated in the presence of palmitoyl-CoA, suggesting a high fat environment may prevent the beneficial effects insulin exerts on mitochondrial bioenergetics.

**RESEARCH DESIGN AND METHODS**

**Ethical**

Male C57Bl6 mice were bred on site at the University of Guelph. Animals were housed in a temperature-controlled environment (22°C) with a 12:12h light-dark cycle. All experimental procedures were approved by the Animal Care Committee at the University of Guelph.

**Experimental design**

Male C57Bl/6N mice (10-15 weeks old) were fed either a control (CTL: 10% kcal from fat, cat. Number D12450J) or high fat (HFD; 60% kcal from fat, cat. Number D12492) diet for 8 weeks (Research Diets, New Brunswick, NJ, USA). All rodents had access to food and water *ad libitum*. Body weight, food and water intake were measured weekly...
and, at the end of the treatment (week 7), indirect calorimetry and glucose tolerance tests were performed (as described below).

After 8 weeks of HFD feeding, and 48 hrs after metabolic characterization, we performed a protocol of acute in vivo insulin stimulation. Specifically, baseline glucose value was obtained and insulin (3 U/kg in 0.9% saline) was injected intraperitoneally twice, 30 minutes apart. We used this approach to guarantee the effects of insulin would still be present even after preparation of permeabilized fibers for mitochondrial bioenergetics assessments. In the non-insulin stimulated mice a similar volume of 0.9% saline was injected intraperitoneally twice (0 and 30 minutes). Thirty minutes after the second injection (1 hr from the first injection), mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg, MTC Pharmaceuticals, Cambridge, ON) and the red gastrocnemius muscle excised for subsequent analysis.

**Glucose tolerance test**

Two days before the acute insulin administration, whole-body glucose tolerance (2 g/kg body weight) was determined by an intraperitoneal glucose tolerance test as previously reported (18).

**Resting whole-body indirect calorimetry**

Resting oxygen consumption (VO\textsubscript{2}) and carbon dioxide production (VCO\textsubscript{2}) were monitored in metabolic caging (Columbus Instruments, Columbus, OH) and used to calculate respiratory exchange ratio as previously reported (19). Indirect calorimetry was performed 5 days before the glucose tolerance test.
In vitro insulin stimulation

In a separate cohort of mice, we determined the direct ability of insulin to stimulate mitochondrial ADP sensitivity in an incubated muscle preparation. Specifically, mice were fed a control or an HFD for 8 weeks. Then, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and the hindlimb was skinned, quickly excised, and placed into a buffer containing 118 mM NaCl, 4.69 mM KCl, 1.18 mM KH$_2$PO$_4$, 1.18 mM MgSO$_4$·7H$_2$O, 24.76 mM NaHCO$_3$, 11.1 mM glucose, and 2.52 mM CaCl$_2$, and 0.3 ng/L tubarine (curare). The buffer was gassed with 95% O$_2$/5% CO$_2$ for 30 minutes, immediately prior to incubations, and the pH was adjusted to 7.2. The Achilles tendon of hindlimbs was severed and pinned open to expose the red gastrocnemius to the incubation buffer. The hindlimb was incubated in the absence (control) or presence of insulin (10 U/L) for 60 minutes. Thereafter, the red gastrocnemius was excised, and fibers were mechanically separated using a microscope and fine-tipped forceps. Fibers were permeabilized with saponin to evaluate mitochondrial bioenergetics (described below).

Preparation of permeabilized muscle fibers

Permeabilized muscle fibers bundles were separated in ice-cold BIOPS using fine-tipped forceps, treated with 40 mg·ml$^{-1}$ saponin for 30 min at 4$^\circ$ C, and then washed in mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM MgCl$_2$·6H$_2$O, 60 mM potassium lactobionate, 10 mM KH$_2$PO$_4$, 20 mM HEPES, 110 mM sucrose, and fatty acid-free bovine serum albumin (BSA; 1 g·l$^{-1}$), osmolarity 330 mOsm, ionic strength 95 mM. Mitochondrial respiration experiments were performed using high-resolution respirometry (Oroboros Oxygraph 2K, Innsbruck, Austria) at 37$^\circ$C in the presence of 5 $\mu$M blebbistatin as previously described (1).
Mitochondrial oxygen consumption

Following the addition of 5 mM pyruvate (cat. number P5280) and 2 mM malate (cat. number 02300), ADP (cat. number A5285) was titrated in various concentrations (from 0 to 10000 µM). 5 mM glutamate (cat. number 49621), 10 mM succinate (cat. number 14160), and 10 µM cytochrome c (to assess mitochondrial membrane integrity, experiments >10% responses were excluded, cat. number C2037) were then sequentially added. ADP titrations were performed in the absence or presence of 60 µM palmitoyl-CoA (P-CoA, cat. number P9716) to model a high intracellular environment (Note: the absence of L-carnitine prevents the transport of P-CoA into the mitochondrial matrix) (1,8, 19). Michaelis-Menten kinetic analysis was used to estimate mitochondrial ADP sensitivity (apparent K_m) whereby O_2 consumption in the absence of ADP (only pyruvate + malate) was considered 0 and analysis was constrained to maximal ADP-supported respiration (100% maximal mitochondrial respiration). All experiments displayed a curve-fit with an R^2 greater than 0.95, and the adjusted R^2 (from 0 to 1) and Sy.x (in pmol · sec^{-1} · mg^{-1} dry wt) are reported in each figure legend to specify the goodness-of-fit. To evaluate the effect of ADP recycling/ATPase activity on mitochondrial respiration, mitochondrial respiration was also measured in the presence/absence of a hexokinase-ADP clamp (0.5 U/mL hexokinase; 3 mM 2-deoxyglucose) as previously described (19).

Western Blotting

The content of insulin signaling proteins were examined by Western blotting in red gastrocnemius samples snap-frozen in liquid nitrogen as previously described (1). Samples were processed and loaded equally using standard SDS-PAGE methodology, transferred to polyvinylidene difluoride membranes, and incubated in the appropriate blocking solution according to the antibody used. Antibodies used in this study include, total-Akt (1:1000, Cell Signaling Technology cat. no. 4691, Danvers, MA, USA),
phosphorylated Akt-Ser473 (1:1000, Cell Signaling Technology cat. no. 9271),
phosphorylated Akt-Thr308 (1:1000, Cell Signaling Technology cat. no. 9275),
phosphorylated JNK1/2 (1:1000, Cell Signaling Technology cat. no. 4671), total JNK1/2
(1:1000, Cell Signaling Technology cat. no. 9252), total p38MAPK (1:1000, Cell
Signaling Technology cat. no. 9212), phosphorylated p38MAPK (1:1000, Cell Signaling
Technology cat. no. 9211), total ERK1/2 (1:1000, Cell Signaling Technology cat. no.
4695), phosphorylated ERK1/2 (1:1000, Cell Signaling Technology cat. no. 9101), total
AMPK (1:1000, Cell Signaling Technology cat. no. 2757), phosphorylated AMPK
(1:2000, Cell Signaling Technology cat. no. 2535), total CaMK-II (1:1000, Cell Signaling
Technology cat. no. 3362), phosphorylated CaMK-II (1:1000, Cell Signaling Technology
cat. no. 12716). All membranes were detected using enhanced chemiluminescence
(ChemiGenius2 Bioimaging System, SynGene, Cambridge, UK). Ponceau staining was
used to confirm consistent loading.

Statistical analysis

Data are expressed as mean ± SD for the kinetic experiments or individual observations
with mean ± SD highlighted. Unpaired two-tailed student’s t-test was used to analyze data
between control and HFD-fed groups. To test the interaction between HFD and insulin,
we used two-way ANOVA followed by LSD post-hoc analysis. Since different cohorts
were used to test specific questions within the study, the specific sample size is provided
in each figure/experimental design. Statistical significance was considered when P<0.05.

RESULTS

Acute insulin stimulation increases mitochondrial ADP sensitivity

We first aimed to determine if acute insulin administration would affect
mitochondrial bioenergetics. Insulin administration decreased blood glucose by almost
50% (Figure 1A). While insulin did not change mitochondrial oxygen consumption in the absence (pyruvate + malate) or presence of maximal ADP (Figure 1B), mitochondrial respiration under submaximal ADP concentrations (175 µM), a situation better resembling a biological environment, was greater after an insulin bolus (Figure 1C). Moreover, ADP titrations in the presence of pyruvate + malate revealed an increase on mitochondrial ADP sensitivity following insulin administration as evident by a reduction in the apparent ADP $K_m$ (Figure 1D, 1E). Importantly, the stimulatory effect of insulin was still present in the presence of a hexokinase clamp (Figure 1B-E), suggesting ATP-consuming cellular processes do not contribute to the increase in oxygen consumption, implicating a direct effect on mitochondrial bioenergetics.

**Verification of the HFD model**

Having observed the ability of insulin to rapidly stimulate mitochondrial ADP sensitivity, we next investigated the potential that HFD consumption, and the induction of insulin resistance, could mitigate the stimulatory effects of insulin. HFD feeding resulted in greater body weight (Figure 2A) and greater glucose area under the curve during ipGTT experiments (Figure 2B, 2C). Moreover, mice exhibited lower respiratory exchange ratio (RER) during both light and dark cycle (Figure 2D), suggesting a preference for whole-body lipid oxidation in HFD-fed mice. Within skeletal muscle, HFD consumption resulted in reduced submaximal (250 µM ADP) but not maximal mitochondrial oxidative phosphorylation (Figure 2E). Although HFD-consumption did not alter the basal phosphorylation status of several key signaling proteins, including Akt (Ser473 and Thr308), CaMK-II phosphorylation was increased in the HFD-fed group (Figure 2F). Combined, these data suggest that 8 weeks of HFD-feeding caused obesity, whole-body glucose intolerance, and impaired submaximal ADP-supported mitochondrial bioenergetics in the absence of insulin.
**Effects of insulin on mitochondrial respiratory capacity following HFD**

Next, we examined the acute effects of insulin on mitochondrial respiration following HFD consumption (Figure 3). HFD-feeding decreased the ability of insulin to stimulate Akt-Thr308, Akt-Ser473, p38MAPK (p=0.05), and CaMK-II phosphorylation (Figure 3A). While leak respiration (State II, PM), maximal oxidative phosphorylation (State III, presence of ADP) (Figure 3B) and the respiratory control ratio in the presence of pyruvate + malate (RCR) (Figure 3C) were not affected by insulin, we reasoned that subtle changes in response to insulin could be lost in the presence of saturating ADP, a situation not present biologically. Surprisingly, despite the induction of insulin resistance (Figure 3A), insulin acutely increased mitochondrial respiration in the presence of submaximal ADP concentrations (< 1000 µM) in both control and HFD-fed groups (Figure 4) when ADP was titrated in the presence of pyruvate + malate. As a result, insulin increased mitochondrial ADP sensitivity by decreasing the apparent ADP K_m by ~30% in the control group and by ~50% in HFD-fed group (Figure 4A-C). Furthermore, under submaximal ADP concentrations (100, 175, 250, and 500 µM), insulin increased (main effect of insulin with no interaction between factors) mitochondrial O_2 consumption almost 50% regardless of the diet (Figure 4D-G). Importantly, when analyzed with a Student t-test as previously reported (1) and depicted in Figure 1, submaximal mitochondrial ADP sensitivity was impaired following HFD consumption, whereas there was no difference between diets in the presence of insulin, strongly suggesting insulin mitigates the deleterious effects of chronic HFD consumption on mitochondrial bioenergetics.

**In vitro effects of insulin on mitochondrial ADP sensitivity**

Since insulin influences the metabolism of glucose, lipids, and amino acids *in vivo*, we decided to test the effects of insulin on mitochondrial bioenergetics in a more controlled
system. To determine this, we excised mouse hindlimbs from control- and HFD-fed mice and incubated muscles in the presence and absence of insulin for 60 minutes to determine the possible direct effect on skeletal muscle mitochondrial ADP sensitivity in the absence of other hormones/organs. Importantly, insulin decreased the apparent ADP $K_m$ ~20% and 40% within the red gastrocnemius of control and HFD-fed mice respectively (Figure 5A-C), indicating a direct effect of insulin on skeletal muscle mitochondrial respiratory function.

**Acute effects of insulin on mitochondrial ADP sensitivity are blunted in the presence of P-CoA**

The previous respiratory experiments were designed to ascertain the independent effects of insulin, and therefore the *in vitro* environment was kept consistent between control and HFD-fed mice. However, ectopic lipid accumulation has been associated with skeletal muscle insulin resistance, and a high lipid environment may interact/attenuate the stimulatory effects of insulin. To model this situation, we determined ADP sensitivity in the presence of P-CoA, as the absence of L-carnitine in our buffer would prevent lipid transport into the mitochondrial matrix, and therefore represent a high cytosolic lipid environment characteristic of HFD. Following HFD consumption, the presence of P-CoA increased the apparent ADP $K_m$ 2-fold to ~1900 µM regardless of the administration of insulin (Figure 6A, B), suggesting that a high cytosolic lipid environment (P-CoA) prevents the beneficial effects of acute insulin on mitochondrial bioenergetics. Importantly, the exogenous provision of cytochrome c did not stimulate respiration in the presence of P-CoA (+P-CoA no Insulin $+7.22 \pm 3.12$; +Insulin $+9.49 \pm 4.40$, %). Furthermore, while insulin increased submaximal (250 µM) ADP-supported mitochondrial respiration after HFD-feeding (Figure 6C), these effects were completely prevented by the presence of P-CoA (Figure 6C). Importantly, insulin failed to stimulate
mitochondrial respiration in the presence of P-CoA when a HK-clamp was utilized during the ADP titration (Figure 6D). These data suggest the effects of insulin on mitochondrial ADP sensitivity are dependent on the absence of P-CoA, a lipid moiety known to inhibit ANT-mediated ADP transport (22). Combined, our results demonstrate that insulin has the capacity to improve mitochondrial ADP sensitivity, even in the presence of HFD-induced insulin resistance. However, the ability of insulin to increase mitochondrial ADP sensitivity is mitigated by the presence of a high intracellular lipid environment (Figure 6A-C).

Finally, to better depict the effects of insulin on mitochondrial bioenergetics we compared our results from insulin-responsive mice in the absence of P-CoA to HFD-fed mice in the presence of P-CoA. Using this approach, the effects of insulin are dramatically different between metabolic environments, and the apparent mitochondrial respiratory dysfunction following HFD is exacerbated. Specifically, while insulin stimulates mitochondrial respiration in control mice, there was virtually no drive in mitochondrial respiration (submaximal 250 µM ADP concentrations) in HFD-fed mice in the presence of P-CoA, creating a ~4-fold reduction in submaximal respiration (Figure 7A). A schematic representation is provided to depict these findings (Figure 7B), as well as a summary schematic to highlight the key finding that insulin acutely increases mitochondrial ADP sensitivity, a response prevented/overridden by the presence of a high-lipid environment (P-CoA) associated with high fat feeding (Figure 7C).

**DISCUSSION**

Mitochondrial dysfunction has been linked to an attenuation in insulin signaling and, in the present study, mitochondrial respiratory function was impaired following HFD in the
absence of insulin. However, we present evidence that insulin acutely increases skeletal muscle mitochondrial ADP sensitivity and submaximal oxidative phosphorylation regardless of HFD-induced insulin resistance. While these data suggest that insulin mitigates the decrease in submaximal respiratory function following HFD consumption, the effects of insulin were lost in the presence of P-CoA. Combined, these data suggest that respiratory function is decreased following HFD, a phenotype that would be exaggerated in a post-prandial state as insulin does not stimulate respiration in the presence of intracellular P-CoA.

**Effects of insulin on mitochondrial ADP sensitivity**

In the present study, our data highlight that insulin acutely increases mitochondrial ADP sensitivity, indicated by a lower ADP $K_m$ and a higher respiration rate in the presence of submaximal ADP and saturating pyruvate + malate. We have only used saturating pyruvate + malate to support our ADP titrations, and it is possible the present findings are specific to these substrate conditions. However, NADH-mediated electron entry through complex I stoichiometrically represents the majority of electrons liberated during substrate oxidation, and therefore likely the most biologically relevant. Moreover, while ADP is known to inhibit pyruvate dehydrogenase kinase (PDK) to stimulate pyruvate dehydrogenase (PDH) *in vivo*, saturating pyruvate before the addition of ADP would also inhibit PDK to activate PDH. While these theoretical arguments implicate ADP transport and/or interaction with ATP synthase as possible explanations for the observed insulin-stimulated increase in submaximal respiration, nevertheless, the present findings may not extend to other substrate conditions.

Since insulin similarly stimulated mitochondrial respiration in the presence of insulin resistance in the HFD-fed group, the insulin-mediated increase in mitochondrial ADP sensitivity might not be related to the canonical insulin signaling pathway. While
the signals regulating mitochondrial ADP sensitivity remain unknown, in the present study, insulin acutely increased CaMK-II phosphorylation suggesting Ca\(^{2+}\) accumulation within the cytosol. Cytosolic Ca\(^{2+}\) concentrations are tightly regulated by Ca\(^{2+}\) efflux through Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) and sarcoplasmic reticulum (SR) reuptake by SR Ca\(^{2+}\) ATPase (SERCA) pumping activity. In this context, it has been shown that insulin elicits a transient increase in [Ca\(^{2+}\)]\(_i\) through ryanodine receptor (RyR) and inositol 1,4,5-triphosphate receptor (IP\(_3\)R) (23). We and others have previously demonstrated that RyR is susceptible to redox modifications which increases the probability of RyR channel opening, resulting Ca\(^{2+}\) leak and CaMK-II activation (24,25). Moreover, SERCA activity is dependent on ATP availability, and ATP consumption by SERCA has been shown to account for ~40-50% of resting metabolic rate in skeletal muscle (26). Since the HK-clamp did not affect mitochondrial respiration in the presence of insulin, it would be reasonable to speculate that insulin directly stimulates mitochondrial bioenergetics as a feed-forward mechanism to prepare for an increase in ATP consumption as a result of a transient Ca\(^{2+}\) influx during acute insulin stimulation. While insulin did not stimulate CaMK-II phosphorylation following HFD consumption, basal CaMK-II phosphorylation levels were already increased following HFD (present study and (25)). It therefore remains possible that insulin increased cytosolic Ca\(^{2+}\) following HFD to stimulate respiration, but it is unlikely to occur directly from CaMK-II activation. An additional hypothesis to consider is that insulin may trigger SR-mitochondrial physical interactions, which would impact energy metabolism and mitochondrial function (27).

Although Akt has been implicated as a central regulator of insulin action, recently, its role has been challenged since chronic skeletal muscle-specific Akt2 ablation did not result in insulin resistance or reductions in glucose uptake (9). Importantly, Akt2 accounts for ~90% of Akt within skeletal muscle, which suggest that either a lower
phosphorylation fraction could be sufficient to drive insulin-stimulated mitochondrial responses or Akt1 could be the mediator of this response. Moreover, based on the findings of triple Akt knock-out, a non-canonical insulin pathway through atypical protein kinase Cλ (aPKCλ) has been proposed (28). In liver, aPKCλ can be activated by IRS-PI3K-PDK1 cascade, in an Akt-independent manner. Moreover, as discussed prior, insulin-stimulated SR Ca2+ release has been proposed to be independent of Akt activation (24), through PLC/IP3R phosphorylation, which could explain the effects of insulin on mitochondrial ADP sensitivity even in HFD-induced insulin resistance when Akt phosphorylation was decreased. Nevertheless, the molecular pathway linking insulin signaling to mitochondrial function remains to be fully elucidated.

Interestingly, Zhao et al., (27) showed that almost half of the mitochondrial phosphorylation sites were identified only under the insulin-stimulated state, including those involved in ATP synthesis and ATP/ADP exchange. Importantly, post-translational regulation of ANT is proposed to influence nucleotide exchange and may be implicated in the reduction in mitochondrial ADP sensitivity following HFD (1). Therefore, it is plausible to speculate that ANT could be influenced by acute insulin exposure, which might explain the observed increase in submaximal mitochondrial O2 consumption. In addition, it seems reasonable that the influence of acute insulin on mitochondrial bioenergetics is a result of several signaling pathways activated in response to glucose uptake. Interestingly, it has recently been reported that higher glycolytic flux can activate mTORC1, a classical downstream target of insulin, in an AMPK-independent manner (30). In our experimental model, the acute insulin bolus did not alter AMPK phosphorylation levels. Notably, pharmacological inhibition of mTORC1 activity reduces mitochondrial O2 consumption in skeletal muscle cells (31,32) whereas it has been shown in L6 skeletal muscle cells culture that the Akt-mTORC1 signaling pathway
is required for the effects of insulin on mitochondrial metabolism and fusion (33).

Nevertheless, a comprehensive investigation of the rapid mechanisms involved in the effects of insulin on mitochondrial bioenergetics requires further investigation. Although the detailed mechanisms were not addressed in the present study, a schematic figure summarizes our main findings and their implications in various physiological states (Figure 7B, 7C). Specifically, under basal conditions of low insulin concentrations (Figure 7C, left panel), the phosphorylation status of insulin targets is low (Figure 2F, 3A), and baseline mitochondria activity is low (Figure 3B, 4D-G). In response to a meal (middle panel), insulin levels increase resulting in an activation of Akt, p38, and CaMK-II (Figure 3A), and an increase in glucose uptake and ATP demand. These signaling and metabolic responses cause an increase in mitochondrial ADP sensitivity, resulting in higher mitochondrial activity that could be related to the maintenance of the ATP/ADP ratio. However, in a high lipid environment (right panel), as observed in HFD-feeding, mitochondrial responses to insulin would be abolished, suggesting lipid-dependent effects of insulin on mitochondrial bioenergetics.

**Effects of lipids on insulin-mediated mitochondrial function**

Excessive lipid availability is suggested as an underlying mechanism driving insulin resistance. Currently, it is extensively debated whether intramyocellular lipid accumulation is a result of mitochondrial dysfunction or rather if these lipids can impair mitochondrial function (4, 5, 26, 27). In the present study, our data highlight the robust negative effects of HFD on mitochondrial bioenergetics, as mitochondrial ADP sensitivity was decreased in HFD-fed mice. These observed effects are strongly related to mitochondrial bioenergetics as opposed to other endogenous ATP-consuming processes, as the addition of blebbistatin prevents myosin ATPase activity, and we have shown using a hexokinase-clamp that changes in ADP sensitivity are not influenced by
ATPase-recycling (present study and (35)). Furthermore, the presence of P-CoA completely mitigated the ability of insulin to stimulate mitochondrial bioenergetics, a process that is directly related to reductions in mitochondrial ADP transport (21). This would suggest an impaired ability for ADP to rapidly modify flux through the electron transport, which has implications to oxidative phosphorylation and redox balance. In further support of our findings that lipids impair mitochondrial bioenergetics, in rat and human myoblasts the insulin-mediated increase in respiratory control is blunted by the presence of palmitate (12). In addition, during a hyperinsulinemic-euglycemic clamp in healthy men, the stimulatory effects of insulin on mitochondrial ATP synthesis was 40% lower when lipids were simultaneously infused (36). While these experimental approaches implicate lipid-mediated signals in the induction of insulin resistance, the present experimental design was focused on determining the direct effects of lipids on mitochondrial bioenergetics, and therefore provides no insight into the chronic effects of ectopic lipid accumulation.

While these studies provide compelling evidence that lipids acutely influence mitochondrial function, it remains debatable exactly how lipids impair respiration. Notably, lipids can directly impair mitochondrial function by binding to ANT and reducing its sensitivity for nucleotide transfer (1,20,21). While this may account for the rapid inhibition of respiration in the presence of P-CoA (in the absence of L-carnitine), but this cannot explain the consistent finding for reduced submaximal ADP-supported respiration following HFD when P-CoA is excluded from the media (1,20), as this would represent a chronic response to the high-fat environment in vivo. While lipids have been suggested to induce uncoupling, this would in contrast be expected to increase oxygen consumption (35), and therefore is not a likely explanation for the present findings. Alternatively, lipids display a high propensity for mitochondrial ROS production when
transferred into the matrix (36) and therefore may also indirectly inhibit ANT, contributing to the reduction in submaximal respiration following HFD in the absence of P-CoA in the respiratory chamber. In support of this, ANT has three known cysteine sites and evidence has accumulated to suggest that ANT is impaired in situations of redox stress (e.g. high fat diet and aging) (1,37). Interestingly, insulin can acutely increase malonyl-CoA concentrations within skeletal muscle (40), a well-known inhibitor of mitochondrial lipid transport, which biologically could contribute to lower mitochondrial ROS production and, as a result, lower ANT cysteine oxidation/increased ADP sensitivity. However, it is also possible a less ‘rigid’ mechanism exists, as lysine 23 acetylation (41), tyrosine 194 phosphorylation (42), and glutathionylation/carbonylation (43) have all been proposed as regulatory mechanisms on ANT. Although speculative, it is therefore possible insulin influences these processes to improve mitochondrial ADP sensitivity. Alternatively, it is also possible that insulin regulates ATP synthase, a mechanism that would occur completely independent of the inhibitory effects of P-CoA. Several post-translational modifications have been reported to alter ATP synthase function (30,44), and, it has been shown that insulin increases ATP synthesis rate within skeletal muscle of lean mice (14). However, post-translational modifications of ATP synthase have previously been shown to alter maximal ADP supported respiration (44), a situation not observed in the present study following insulin administration. Alternatively, HFD and lipid-induced impairments in mitochondrial ADP sensitivity could be interpreted as an adaptation to maintain some glycolytic flux by accumulating cytosolic ADP; however, at the expense of greater mitochondrial ROS production. Although the present data suggests a high lipid environment prevents insulin-mediated improvements in mitochondrial function, the interplay between lipids, insulin, and mitochondrial function warrants further investigation.
Conclusion

The notion that impaired mitochondrial bioenergetics contributes to the induction of insulin resistance has been extensively studied, however in the present study we provide evidence that insulin rapidly regulates mitochondrial bioenergetics by increasing ADP respiratory sensitivity. Specifically, we show that i) insulin rapidly increases submaximal oxidative phosphorylation, and while ii) HFD impairs submaximal ADP-supported respiration, this HFD-mediated impairment in mitochondrial bioenergetics is iii) completely prevented by insulin, despite the apparent reduction in insulin signaling. However, the beneficial effects of insulin were iv) mitigated by the presence of P-CoA, suggesting the high lipid environment related to HFD prevents the ability of insulin to stimulate oxidative phosphorylation. Combined, these data provide compelling evidence that mitochondrial bioenergetics are impaired following HFD, as in the basal state (absence of insulin) submaximal respiration is impaired, and in the post-prandial state (presence of insulin) a high lipid environment prevents the effects of insulin.

Data Availability Statement: Full data will be provided upon reasonable request.

Conflict of interest: The authors declare no conflict of interest in this study.

Dr. Graham P. Holloway is the guarantor of this study.

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Figure 1 – Insulin rapidly increases mitochondrial ADP sensitivity. Blood glucose values after insulin injections (A, n=5-7/group), maximal respiratory capacity (B, n=4-6/group), submaximal ADP-supported mitochondrial respiration (175 µM ADP) (C, n=4-6/group), ADP titration (D, n=4-6/group; adjusted R² for CTL – Ins = 0.99; CTL + Ins = 0.98; +HK – Ins = 0.97; +HK + Ins = 0.98; Sy.x for CTL – Ins = 2.89; CTL + Ins = 3.79; +HK – Ins = 5.15; +HK + Ins = 3.81 pmol · sec⁻¹ · mg⁻¹ dry wt), and apparent ADP sensitivity (E, n=4-6/group). P – pyruvate; M – malate; D – adenosine diphosphate; G – glutamate; S – succinate; C – cytochrome c; CTL – control group; HK – hexokinase clamp condition; J02 – O2 consumption rate; Km – Michaelis-Menten constant; Data are shown as individual observations with mean ± SD superimposed. * P<0.05. Statistical analysis: two-way ANOVA with LSD post-hoc test.

Figure 2 - Metabolic characterization of the HFD-fed mice. Body weight gain (A, n=8/group), glucose tolerance (B, n=5/group) and area under the curve calculated from ipGTT (C, 5/group), respiratory exchange ratio (D, n=4-5/group), mitochondrial respiration (E, n=7-9/group), and baseline protein phosphorylation levels (F, n=6-7/group). CTL – control; HFD – high-fat diet; RER – respiratory exchange ratio; AU – arbitrary units; P – pyruvate; M – malate; D – adenosine diphosphate. Data are shown as individual observations with mean ± SD superimposed. * P<0.05. Statistical analysis: Two-tailed unpaired student t-test.

Figure 3 – Effects of HFD and insulin on mitochondrial content and maximal respiratory capacity. Protein phosphorylation levels (A, n=7/group) after insulin stimulation, maximal mitochondrial respiratory capacity (B, n=9-14/group), respiratory control ratio (C, n=9-14/group) in permeabilized muscle fibers after chronic HFD-feeding and acute insulin stimulation. P – pyruvate; M – malate; D – adenosine diphosphate; G – glutamate; S – succinate; C-II – complex II; C-IV – complex IV; C-V – complex V; CTL – control group; HFD – high-fat diet-fed group; J02 – O2 consumption rate. Data are shown as individual observations with mean ± SD superimposed. Statistical analysis: Two-tailed unpaired student t-test (A) and two-way ANOVA with LSD post-hoc test (B, C).
Figure 4 - Mitochondrial ADP sensitivity and submaximal ADP-supported respiration. ADP titration in control (A, n=14/group; adjusted R² for CTL – Ins = 0.96; CTL + Ins = 0.95; Sy.x for CTL – Ins = 7.73; CTL + Ins = 8.75) and HFD-fed (B, n=14/group; adjusted R² for HFD – Ins = 0.96; HFD + Ins = 0.94; Sy.x for HFD – Ins = 7.28; HFD + Ins = 9.28 pmol · sec⁻¹ · mg⁻¹ dry wt) groups, apparent ADP sensitivity (C, n=14/group), and submaximal ADP-supported mitochondrial respiration (D-G, n=9-11/group) after acute insulin stimulation in control and HFD-fed mice. CTL – control; HFD – high-fat diet; ADP – adenosine diphosphate; Km – Michaelis-Menten constant; JO₂ – O₂ consumption rate. Data are shown as individual observations with mean ± SD superimposed. * P<0.05 compared to non-insulin control-fed group; # P<0.05 compared to non-insulin within the same diet. Data were analyzed with two-way ANOVA with LSD post-hoc test.

Figure 5 – In vitro insulin incubation. ADP titration in red gastrocnemius muscles from CTL-fed mice (A, n=4-5; adjusted R² for CTL – Ins = 0.98; CTL + Ins = 0.96; Sy.x for CTL – Ins = 5.32; CTL + Ins = 6.46), HFD-fed mice (B, n=3; adjusted R² for HFD – Ins = 0.94; HFD + Ins = 0.97; Sy.x for HFD – Ins = 9.47; HFD + Ins = 5.98 pmol · sec⁻¹ · mg⁻¹ dry wt), and apparent ADP sensitivity (C) in the absence and presence of insulin for 60 minutes. CTL – control; HFD – high-fat diet; ADP – adenosine diphosphate; Km – Michaelis-Menten constant; JO₂ – O₂ consumption rate. Data are shown as individual observations with mean ± SD superimposed. * P<0.05. Data were analyzed with two-way ANOVA with LSD post-hoc test.

Figure 6 – Effects of insulin on mitochondrial ADP sensitivity are blunted in the presence of P-CoA. Following 8 weeks of HFD-feeding, ADP titrations were performed in the presence and absence of P-CoA after acute insulin stimulation (A, n=7-10/group, adjusted R² -P-CoA = 0.98; -P-CoA + Ins = 0.96; +P-CoA – Ins = 0.98; +P-CoA + Ins = 0.98; Sy.x -P-CoA = 4.66; -P-CoA + Ins = 6.83; +P-CoA – Ins = 3.98; +P-CoA + Ins = 4.61 pmol · sec⁻¹ · mg⁻¹ dry wt) to determine the apparent ADP sensitivity (B, n=7-10/group). Absolute mitochondrial respiration ADP-stimulated (250µM ADP) after insulin stimulation in the presence or absence of P-CoA (C, n=4-9/group) in HFD-fed mice; Effect of ADP recycling by exogenous hexokinase clamp on mitochondrial ADP sensitivity in the presence of P-CoA (D, n=3/group). CTL – control; HFD – high-fat diet; ADP – adenosine diphosphate; JO₂ – O₂ consumption rate; P-CoA – palmitoyl-CoA. Data are shown as individual observations with mean ± SD superimposed. * P<0.05 compared to control group. Data were analyzed with two-way ANOVA with LSD post-hoc test.

Figure 7 - Schematic figure summarizing the effects of insulin in insulin-sensitive and insulin-resistant skeletal muscle. Absolute mitochondrial (250µM ADP) respiration in control-fed mice after insulin bolus and HFD-fed mice after insulin bolus in the presence of P-CoA (A, n=11/group). Representative kinetic curve lines of best fit depicting mitochondrial ADP kinetics in response to insulin and P-CoA (B). Schematic figure summarizing the effects of insulin on mitochondrial bioenergetics in several conditions (C). Data are shown as individual observations with mean ± SD superimposed. * P<0.05. Data were analyzed unpaired student t-test (A). ADP – adenosine diphosphate; ATP – adenosine triphosphate; CaMK-II - Ca²⁺/calmodulin-dependent protein kinase II; p38MAPK - p38 mitogen-activated protein kinases; SERCA - sarco/endoplasmic reticulum Ca²⁺-ATPase. Created with BioRender.com.
Submaximal ADP concentrations

| ADP Concentration | CTL + Insulin | HFD + Insulin | CTL + Saline | HFD + Saline |
|-------------------|---------------|---------------|--------------|--------------|
| 100 µM            | - Insulin     | + Insulin     | - Insulin    | + Insulin    |
|                   | **p=0.001**   | **p=0.002**   |              |              |
| 175 µM            | - Insulin     | + Insulin     | - Insulin    | + Insulin    |
|                   | **p=0.001**   | **p=0.002**   |              |              |
| 250 µM            | - Insulin     | + Insulin     | - Insulin    | + Insulin    |
|                   | **p=0.001**   | **p=0.002**   |              |              |
| 500 µM            | - Insulin     | + Insulin     | - Insulin    | + Insulin    |
|                   | **p=0.001**   | **p=0.002**   |              |              |
In vitro insulin incubation

A 

B

C

Apparent $K_{m}$ (μM ADP)

- Insulin  
+ Insulin

Main effect

Insulin $p=0.0327$
