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Research Article

Insulin selectively reduces mitochondrial uncoupling in brown adipose tissue in mice

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The purpose of the present study was to determine the effects of prolonged hyperinsulinemia on mitochondrial respiration and uncoupling in distinct adipose tissue depots. Sixteen-week-old male mice were injected daily with placebo or insulin to induce an artificial hyperinsulinemia for 28 days. Following the treatment period, mitochondrial respiration and degree of uncoupling were determined in permeabilized perirenal, inguinal, and interscapular adipose tissue. White adipose tissue (WAT) mitochondria (inguinal and perirenal) respire at substantially lower rates compared with brown adipose tissue (BAT). Insulin treatment resulted in a significant reduction in mitochondrial respiration in inguinal WAT (iWAT) and interscapular BAT (iBAT), but not in perirenal WAT (pWAT). Furthermore, these changes were accompanied by an insulin-induced reduction in UCP-1 (uncoupling protein 1) and PGC-1α in iWAT and iBAT only, but not in pWAT or skeletal muscle. Compared with adipose tissue mitochondria in placebo conditions, adipose tissue from hyperinsulinemic mice manifested a site-specific reduction in mitochondrial respiration probably as a result of reduced uncoupling. These results may help explain weight gain so commonly seen with insulin treatment in type 2 diabetes mellitus.

Introduction

Insulin elicits countless reactions in all cells of the body, thematically centered on adjusting utilization of nutrient substrate for the sake of cellular anabolism. Adipose tissue is particularly responsive to insulin, where insulin activates enzymes essential to lipogenesis [1] and adipogenesis [2], while potently inhibiting lipolysis [3], even when partially insulin-resistant [3]. Interestingly, some of the adipose-specific effects of insulin pertain to both the predominant white and the lesser brown adipose tissue (BAT) depots, despite their very different roles.

Though we now have a greater appreciation for its role as an endocrine organ, white adipose tissue (WAT) is nevertheless accurately primarily considered an energy storage depot. In contrast, BAT, which exists in a few distinct sites on the body [4], is a much more metabolically active organ essentially involved in thermogenesis. The disparate roles (i.e. storage vs. use) for two similar organs (i.e. both adipose tissues) are largely a consequence of very different mitochondrial profiles; WAT, involved principally in energy storage, has a significantly smaller mitochondrial presence than BAT, which is involved more in energy use [5]. However, while we have a clear understanding of the effects of insulin on adipose tissue mass (via anabolism), the effects of insulin on adipose tissue mitochondrial function remain unclear.

That insulin is involved in BAT physiology is established; indeed, its role is both local and central. Locally, the loss of the BAT insulin receptor leads to a reduction in BAT mass [6], suggesting that, like WAT, insulin is necessary, at some point, for BAT development. Centrally, insulin signaling in the

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brain similarly serves to support BAT by increased sympathetic nervous outflow [7]. However, despite these advances, the effects on insulin on WAT and BAT mitochondrial function remain largely unknown.

Insulin has long been known to influence metabolic function, even rate, in humans; Considering that insulin therapy has a stifling effect on metabolic rate in type 1 and 2 diabetics [8–10], as well as the role of BAT in setting metabolic tone, we sought to determine the consequences of insulin *in vivo* on BAT and WAT mitochondrial activity, with a particular focus on the degree of changes in uncoupling proteins and respiration. These results will provide insights into a potential mechanism of the metabolic-dampening effect in states of elevated insulin.

Materials and methods

Animals

Sixteen-week-old male C57Bl/6 mice, housed at 22°C, were separated into one of two groups (six per group) to receive morning injections of either saline (PBS) or insulin (daily; 0.75 U/kg/BW; Actrapid; Novo Nordisk, Plainsboro, NJ, U.S.A.) for 28 days with free access to chow (Harlan 8604) and water throughout the length of the study. Serum insulin (ELISA; Crystal Chem. Inc.) and blood glucose (Bayer OneTouch) were determined. HOMA-IR was determined from fasting glucose and insulin accordingly to the formula: HOMA-IR = insulin (mU/l) × glucose (mg/dl)/405 [11,12]. Blood samples were not taken within 24 h of last insulin injection. Studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC (Institutional Animal Care and Use Committee) at Brigham Young University.

Protein analysis

Proteins were analyzed by western blot, as described previously [13]. Briefly, after lysing and quantification of protein (BCA protein assay; Pierce), 50 μg of protein for each sample, with comparable volume of sample buffer, was loaded into a gel. Samples were then transferred onto the nitrocellulose membrane and immunoblotted with the following antibodies: PGC-1α (Abcam, ab54481); UCP-1 (Cell Signaling, 14 670); β-actin (Cell Signaling, 3700).

Adipose and muscle tissue permeabilization

Tissue was quickly removed from killed mice and immediately placed in ice-cold buffer X [60 mM K-MES, 35 mM KCl, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 20 mM tuarine, 5.7 mM ATP, 15 mM PCr, 6.56 mM $MgCl_2{\cdot}6H_2O$ (pH 7.1)] and trimmed of connective tissue. Following the removal of connective tissue, small (10–20 mg) samples of the adipose tissue were then transferred to a tube with chilled buffer X and 50 μg/ml saponin and rocked at 4°C for 30 min, then washed in buffer Z [105 mM K-MES, 30 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2{\cdot}6H_2O$, 0.5 mg/ml BSA (pH 7.1)] at 4°C for at least 15 min. Samples were then blotted dry and weighed.

Mitochondrial respiration protocol

High-resolution O_2 consumption was determined at 37°C in permeabilized cells and fiber bundles using the Oroboros O2K Oxygraph with MiR05 respiration buffer, as described previously [14–16]. Briefly, before the addition of sample into respiration chambers, a baseline respiration rate was determined. After the addition of sample, the chambers were hyperoxygenated to ∼250 nmol/ml. Following this, respiration was determined by all or parts of the following substrate–uncoupler–inhibitor–titration protocol: electron flow through complex I was supported by glutamate + malate (10 and 2 mM, respectively) to determine leak oxygen consumption (GM_L). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GM_P). Succinate was added (GMS_P) for complex I + II electron flow into the Q-junction. To determine full electron transport system capacity in cells over oxidative phosphorylation, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 μM, followed by 0.025 μM steps until maximal O_2 flux was reached; GMS_E). Mitochondrial membrane integrity was tested in all experiments by adding cytochrome *c* (not shown; 10 μM). Finally, residual oxygen consumption was measured by adding antimycin A (2.5 μM) to block complex III (CIII) action, effectively stopping any electron flow, which provides a baseline rate of respiration. Respiratory control ratio (RCR) and CII factor (CII Factor) were determined by the ratio of GM_P:GM_L and the difference between $GMS_P - GM_P$, respectively.

Biochemical Journal (2018) **475** 561–569
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Indirect calorimetry

The rodent metabolic rate was measured by indirect calorimetry using open-circuit oxymax chambers (Columbus Instruments, Columbus, OH, U.S.A.). Mice were individually housed in a temperature-controlled environment (24°C) with a 12 : 12-h light–dark cycle (light period 0800–2000). Food and water were available *ad libitum*. Following weight recordings, mice were acclimatized to monitoring cages for 24 h prior to 24 h of hourly recordings. O_2 consumption was determined by measuring oxygen concentration in air entering the chamber compared with air leaving the chamber with all calibrations being performed according to the manufacturer. Activity was determined by the number of infrared beam breaks along the *x*-axis of the metabolic cage. Food consumption was tracked by in-cage monitoring.

Statistics

Data are presented as the mean ± SEM. Data were compared by *T*-test or ANOVA with Tukey's *post hoc* analysis (Graphpad Prism, La Jolla, CA, U.S.A.). Significance was set at $P < 0.05$.

Results

Insulin increases body mass and selective fat depots

Our initial finding with 28 days of insulin treatment was an increase in body mass (Figure 1A). In particular, while insulin treatment increased perirenal (pWAT) and inguinal WAT (iWAT), it had no effect on interscapular BAT (iBAT) (Figure 1B). This difference persisted even after controlling for total body mass (Figure 1C). Furthermore, gastrocnemius mass was slightly, yet significantly lower in the insulin (INS)-treated group, when controlled for by body mass (Figure 1C). Importantly, food intake between the two groups did not differ significantly (Figure 2A). However, oxygen consumption, used to determine indirect calorimetry, revealed that chronic hyperinsulinemia significantly depressed energy expenditure during the 'active' night phase of a 24-h light cycle (Figure 2B), with a 17% lower total 24-h energy expenditure. Ambulatory activity (*x*-axis beam breaks) was similar across treatments (Figure 2C).

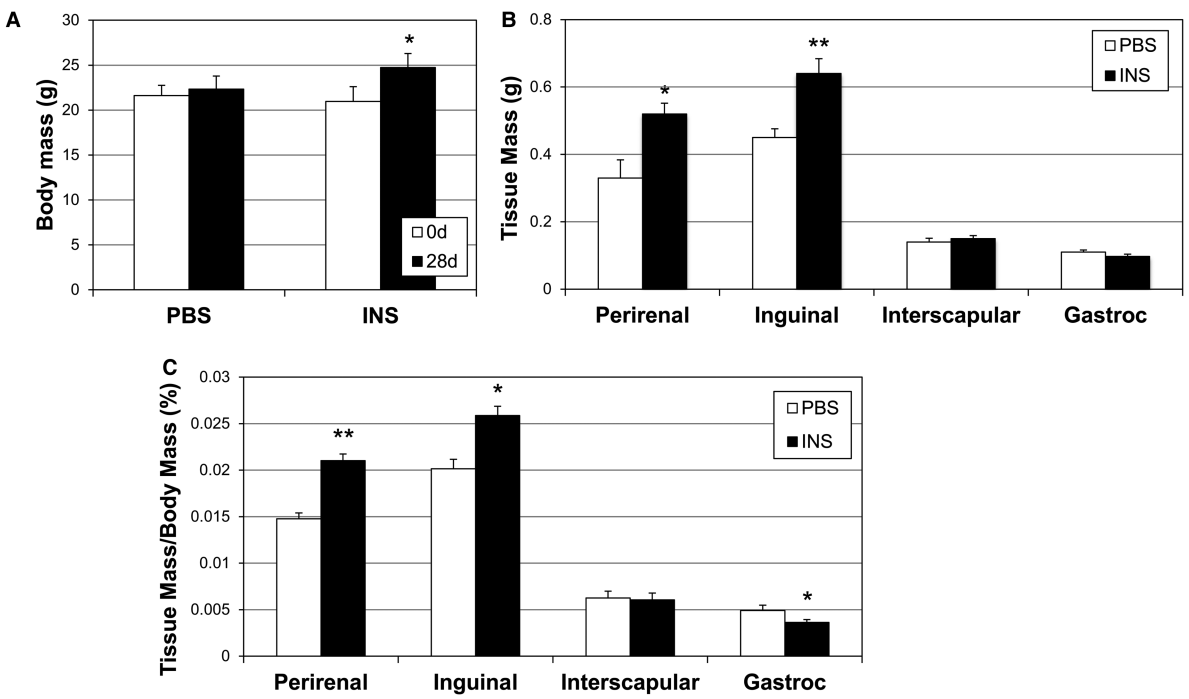

Figure 1. Insulin injections increase body mass and fat mass in mice.

Sixteen-week-old male mice received injections of PBS (daily) or insulin (INS; daily; 0.75 mg/kg) for 28 days.Body mass (**A**; $n = 6$), perirenal, inguinal, and interscapular fat pad mass and gastrocnemius mass were measured (**B**; $n = 6$) and compared with body mass (**C**; $n = 6$). *$P < 0.05$; **$P < 0.01$.

Biochemical Journal (2018) **475** 561–569
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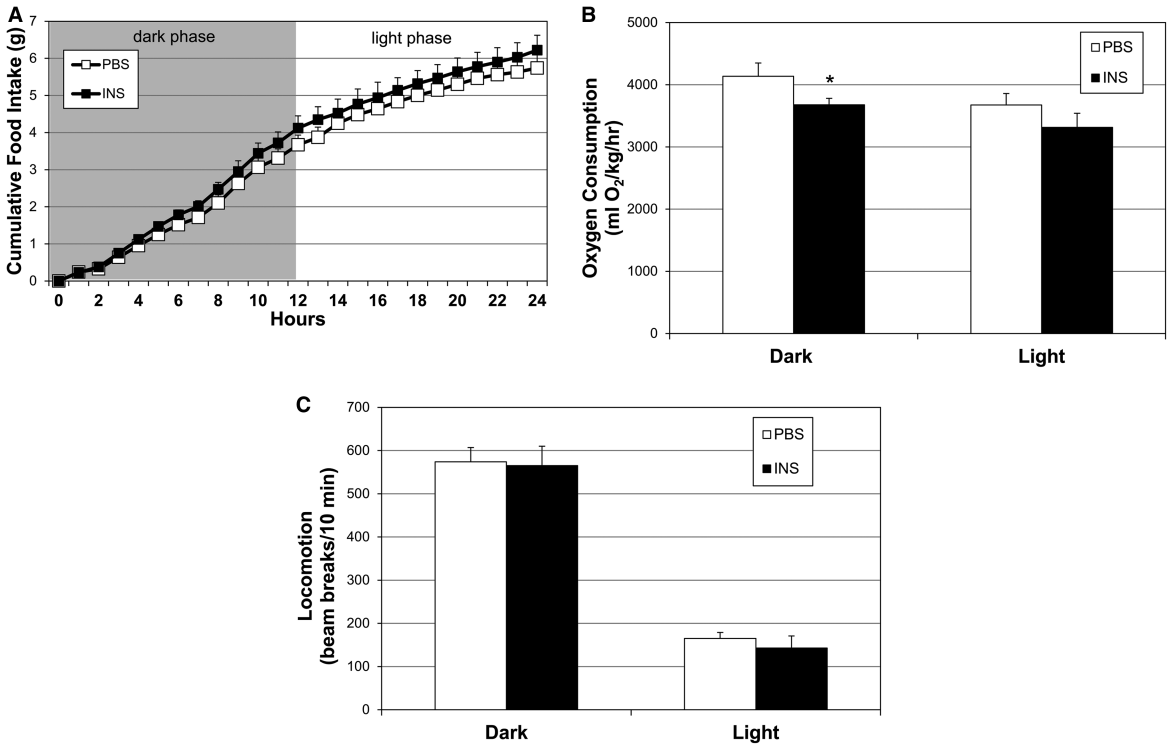

Figure 2. Insulin injections depress oxygen consumption.

At the conclusion of 28 days of intervention, food intake (**A**), O_2 consumption (**B**), and activity (**C**) were analyzed over a 24-h period in PBS- and INS-injected mice ($n = 6$). *$P < 0.05$.

Insulin increases insulin resistance

Following chronic treatment with insulin, fasting blood glucose was measured prior to sacrifice and plasma insulin was determined by ELISA. Unsurprisingly, chronic insulin therapy resulted in increased fasting insulin (Figure 3A). Furthermore, INS-treated rodents also became significantly insulin-resistant, as determined by the HOMA-IR score (Figure 3B). Blood samples were taken at least 24 h following the final insulin injection to ensure assayed plasma insulin levels were a result of endogenous production.

Specific fat depot mitochondria respond disparately to hyperinsulinemia

Mitochondrial respiration was determined in excised pWAT, iWAT, and iBAT in PBS and INS mice. Insulin therapy had no effect on any measurement of oxygen consumption in pWAT (Figure 4A–C). In contrast, insulin injections resulted in specific significant reductions in mitochondrial respiration in iWAT (Figure 4D–F) and iBAT (Figure 4G–I). Specifically, iWAT mitochondria respired at slightly depressed rates during the leak state (GM_L), phosphorylation with glutamate, malate, and succinate (GMS_P). Respiratory control ratio was analyzed *post hoc* as an indicator of overall mitochondrial health [17,18], with no differences across the tissues (Figure 4B,E,H). The addition of succinate to engage CII of the electron transport system elicited a significantly robust response in the iWAT (Figure 4F) and iBAT (Figure 4I), but not in pWAT (Figure 4C).

Insulin discriminately lowers PGC-1α and UCP-1 in adipose tissue depots, but not in muscle

In a similar manner to mitochondrial respiration, insulin treatment had no effect on PGC-1α and UCP-1 protein expression (Figure 5A–C). In contrast, both iWAT and iBAT experienced a reduction in these proteins in the INS group (Figure 5D–I), suggesting an overall depression in mitochondrial biogenesis and uncoupling. Furthermore, we found that hyperinsulinemia elicited a slight, but significant reduction in red gastrocnemius ADP-supported respiration (Figure 6A; GM_P), evidenced by a reduced RCR (Figure 6B). Nevertheless, CII appeared unaffected (Figure 6C), as well as PGC-1α and UCP-1 (undetectable) protein levels (Figure 6D,E).

Biochemical Journal (2018) **475** 561–569
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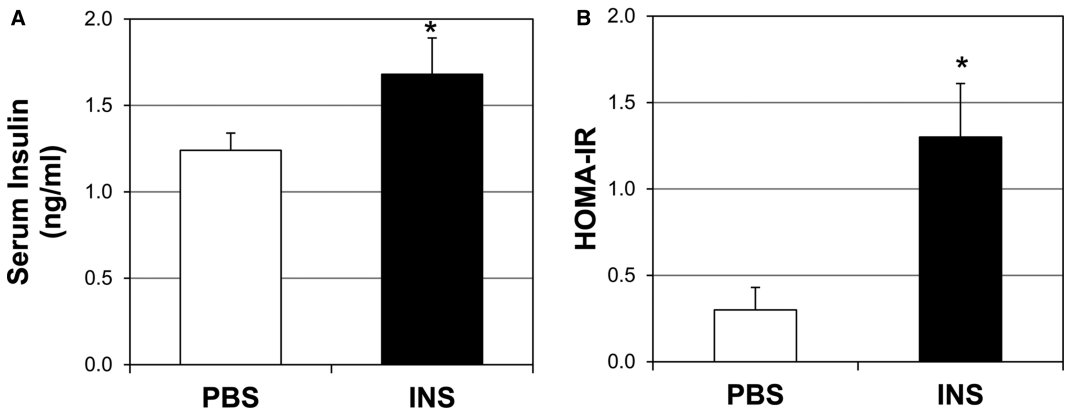

Figure 3. Insulin injections increase insulin resistance in mice.
Following 28 days of insulin injections, fasting (6 h) serum insulin (**A**; $n = 5$) and HOMA-IR were assayed (**B**; $n = 5$).

Discussion

The key finding of these studies is the revelation that prolonged hyperinsulinemia elicits a disparate effect on adipocyte mitochondrial function. Specifically, insulin treatment inhibited mitochondrial uncoupling within prototypical BAT (i.e. interscapular; iBAT) and WAT capable of 'browning' (i.e. inguinal adipose; iWAT), but

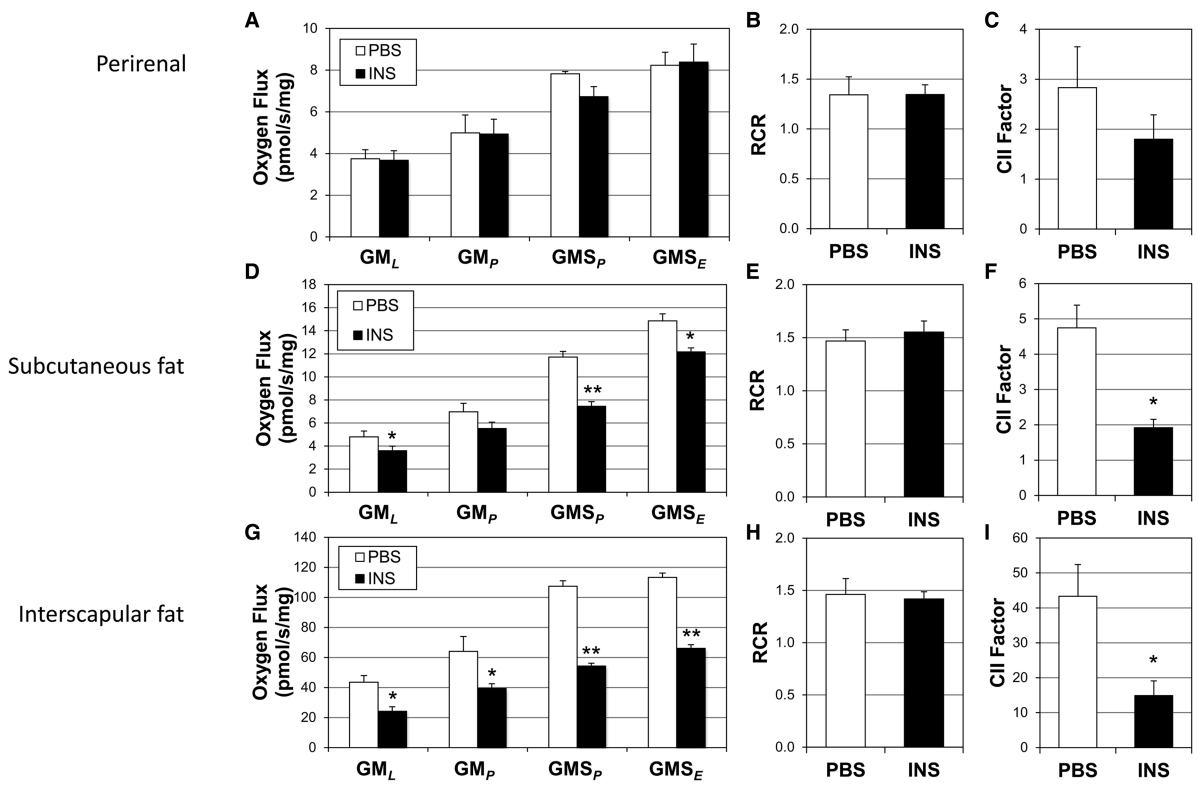

Figure 4. Chronic insulin injections elicit distinct effects on adipocyte mitochondrial function.
Mitochondrial assessments were determined in permeabilized (saponin, 50 μg/ml) adipose tissue following 28 days of treatment. To measure mitochondrial respiration in perirenal (**A**), inguinal (**D**), and interscapular (**G**) adipose tissues, samples were sequentially treated with GM_L: glutamate (10 mM) + malate (2 mM); GM_P: +ADP (2.5 mM); GMS_P: +succinate (10 mM); GMS_E: +FCCP (0.05 μM). RCR (**B**, **E**, and **H**) and CII factor (**C**, **F**, and **I**) were determined in perirenal (**A–C**), inguinal (**D–F**), and interscapular (**G–I**) adipose, respectively, by the analysis indicated in Materials and methods. $N = 6$. $^*P < 0.05$; $^{**}P < 0.01$.

Biochemical Journal (2018) **475** 561–569
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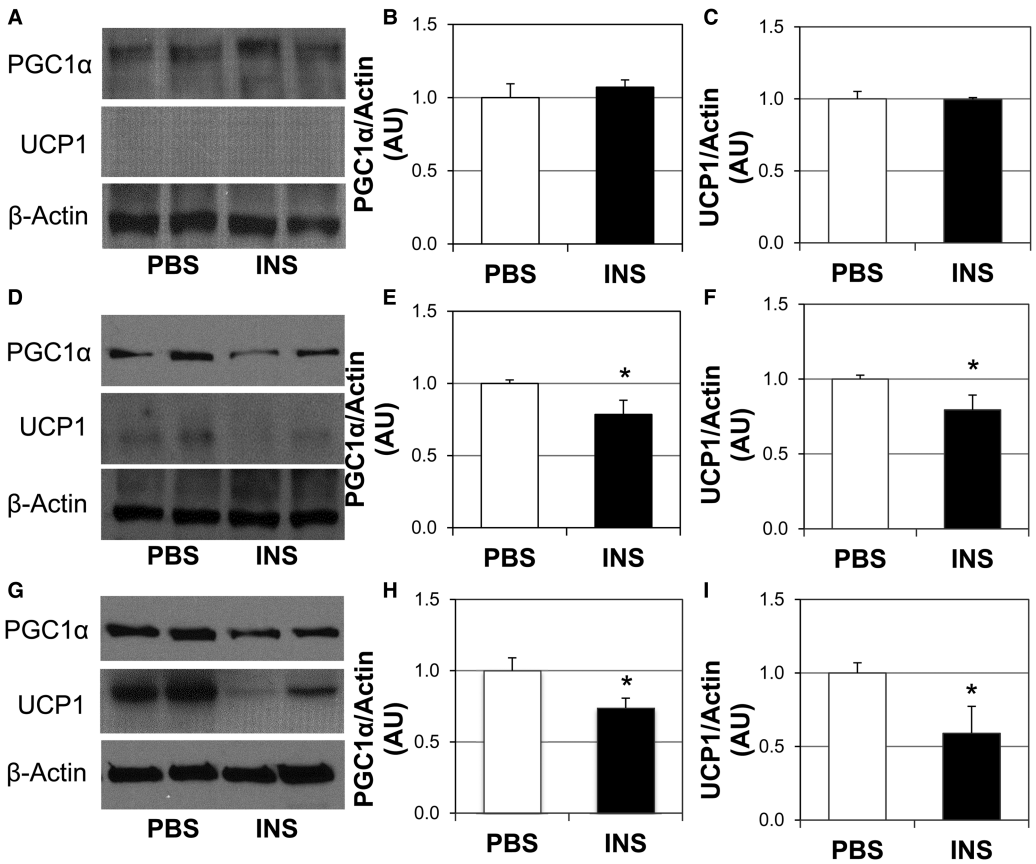

Figure 5. Chronic insulin injections alter adipocyte protein levels.

At the conclusion of the study, PGC-1α and UCP-1 protein levels were determined via Western blot and quantified in perirenal adipose tissue (**A–C**), subcutaneous adipose tissue (**D–F**), and interscapular adipose tissue (**G–I**). $N = 6$. $^*P < 0.05$.

not classic WAT (i.e. perirenal; pWAT). This finding is supported by a significant reduction in mitochondrial respiration in iWAT and iBAT with prolonged insulin treatment, as well as a loss of UCP-1 and PGC-1α protein expression.

Although our studies did not include a temperature component, it is noteworthy that the animals were housed at conventional temperatures (i.e. 22°C), not true thermoneutrality for rodents (i.e. 29°C). This may be relevant insofar as Bal et al. [19] found that mild cold exposure (i.e. 16°C) was sufficient to reduce plasma insulin levels to below 50% compared with mice housed at thermoneutrality. While the effects of housing at conventional 22°C on insulin are unknown, it is possible that all the rodents involved in this study experienced a degree of temperature-induced changes in insulin and, thus, adipocyte mitochondrial uncoupling. However, given that this was common to all animals, we submit that the effect of induced hyperinsulinemia on adipocyte mitochondrial coupling status is nevertheless valid.

While our findings of contrasting mitochondrial responses to insulin within distinct adipose tissue depots in mice are novel, our work corroborates that by Kraunsøe et al. [20], who similarly reported distinct mitochondrial responses between human subcutaneous and visceral adipose tissue. Indeed, we found that iBAT mitochondrial oxygen consumption is over 10-fold higher than that of pWAT and almost as high as that of iWAT. Importantly, the functionality, or 'fitness', of the mitochondria appears to be similar across the various fat tissues, as evidenced by comparable levels and changes in the respiratory control ratio [17,18]. However, notable differences were observed in analyzing CII factor, a simple comparison between respiration rates prior to and following the addition of succinate. The pWAT from INS-treated mice had no significant increase in respiration with the addition of succinate; however, the difference was robust in iWAT (43%) and iBAT (36%).

Our mitochondrial findings provide an interesting bioenergetic compliment to those of Boucher et al. [21], who recently revealed that mice lacking adipose tissue insulin receptors have a disparate effect in regulating

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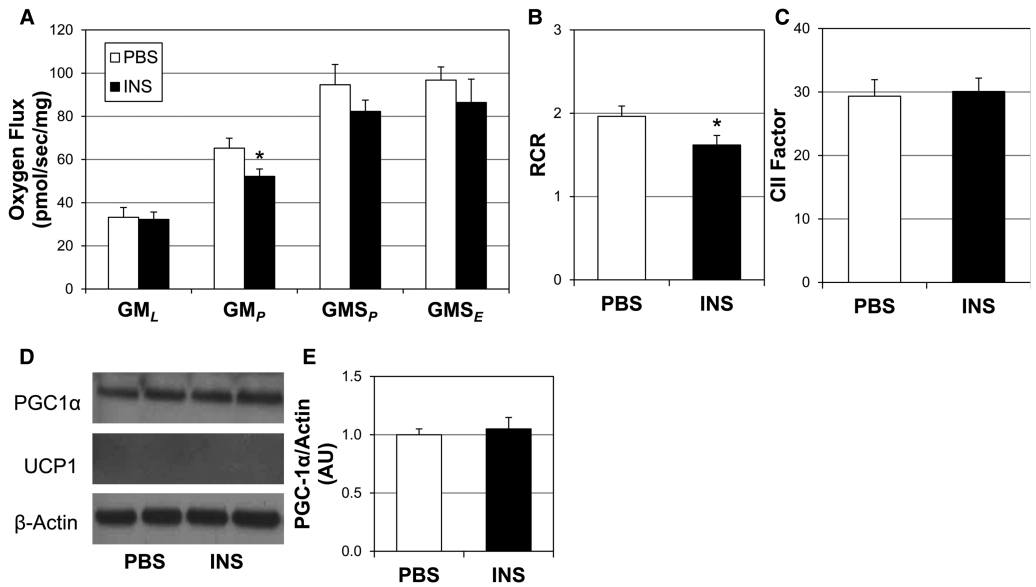

Figure 6. Chronic insulin injections alter skeletal muscle mitochondrial respiration.

At the conclusion of the study, mitochondrial assessments were determined in permeabilized (saponin, 50 μg/ml) red gastrocnemius from control (PBS) and insulin-treated (INS) mice. To measure respiration in the tissue (**A**), samples were sequentially treated with GM_L: glutamate (10 mM) + malate (2 mM); GM_P: +ADP (2.5 mM); GMS_P: +succinate (10 mM); GMS_E: +FCCP (0.05 μM). RCR (**B**) and CII factor (**C**) were determined by the analysis indicated in Materials and methods. PGC-1α and UCP1 protein levels were determined via Western blot (**D**) and quantified (**E**; no quantification for UCP-1). $N = 6$. $^*P < 0.05$.

white vs. brown adipose; specifically, lack of insulin signaling results in diminished white adipose mass, but increased brown adipose mass. Additionally, Mehran et al. [22] found that insulin was a critical regulator of adipocyte UCP-1 expression. Overall, our collective findings suggest an important role for insulin in the maintenance of WAT, but not of BAT mitochondrial physiology. Furthermore, we have shown previously that insulin increases tissue ceramide accrual [15,23], and Chaurasia et al. [24] reveal that inhibition of ceramide biosynthesis increases adipocyte mitochondrial uncoupling. Combined, these results elicit the tempting conclusion that adipocyte ceramide accrual may be a mediator of the insulin-induced adipocyte mitochondrial changes we observed. Future efforts will confirm the validity of this speculation.

We believe that these findings are relevant to our general understanding of obesity. The prevailing and calorie-centric focus on obesity have several relevant considerations in the determination of energy balance, including basal metabolic rate, physical activity, the thermic effect of food and, more recently, metabolically active BATs [25,26]. Long known to influence rodent metabolic function, the role of BAT in adult human physiology has been controversial [27], though recent evidence not only suggests BAT present in adult humans but also elicits demonstrable and even relevant effects on human adiposity [26,28]. However, while the focus on obesity as a caloric imbalance certainly has merit, the endocrine component of the condition must also be considered to fully understand the condition and improve interventions. Elevated insulin levels are a fundamental feature of obesity [29–33]. In addition to potently inhibiting lipolysis [3], insulin promotes hepatic [34] and adipocyte lipogenesis [1], as well as adipogenesis [2]. Furthermore, insulin treatment invariably increases weight gain in type 2 diabetics, even when caloric consumption is reduced [9], and higher insulin dosing in type 1 diabetics leads to greater weight gain [10]. We have also shown previously that prolonged insulin treatment induces weight gain and insulin resistance in animals [15,23], an effect similar to that seen in humans [35]. Notably, these results are relevant in the rich history of research exploring the metabolism of diabetes. Specifically, noteworthy diabetes and metabolic scientists Elliot P. Joslin and Francis G. Benedict noted (in 1912) that metabolic rate in untreated insulin-deficient diabetes was roughly 15% higher compared with similar body weight subjects with normal insulin [36].

Interestingly, the two theories of obesity, namely endocrine and caloric, are perhaps not mutually exclusive; insulin itself may represent a unifying paradigm that considers both aspects of obesity. Insulin clearly

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influences fuel use by regulating adipocyte lipid metabolism, but these effects are supplemented by observations that, even when fed isocalorically, diets that maintain lower levels of insulin not only promote greater fat loss in humans [37,38] but also increase UCP-1 expression in BAT in mice [39], findings that have yet to be studied in humans. Altogether, these results serve to promote a unifying theory with insulin as the mediator.

Conclusions

In conclusion, previous reports have predominately added evidence to one of two theories of the origins of obesity — the number of calories vs. the endocrine response to calories. Our findings of insulin eliciting a demonstrable mitochondrial effect in adipose tissue, namely a reduction in uncoupling, provide, to a degree, a fusion of ideas. Namely, insulin activates enzymatic processes involved in adipose lipid storage and reduces unnecessary energy expenditure in adipose tissue, lowering metabolic rate. Such conclusions echo earlier sentiments of a preeminent scientist, Dr George Cahill, who considered insulin the key to 'fuel control in mammals' [40].

Abbreviations

BAT, brown adipose tissue; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; HOMA-IR, homeostatic model assessment-insulin resistance; iBAT, interscapular BAT; INS, insulin; iWAT, inguinal WAT; pWAT, perirenal WAT; RCR, respiratory control ratio; UCP, uncoupling protein; WAT, white adipose tissue.

Author Contribution

B.T.B. conceived and managed the study. B.W.D., B.A.P., A.E.H., T.S.T., M.E.H., M.M.A.A., J.E.W., J.L.G., H.M. G. and B.T.B. performed and analyzed all experiments. B.W.D. and B.T.B. prepared the manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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