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Insulin-stimulated mitochondrial adenosine triphosphate synthesis is blunted in skeletal muscles of high-fat–fed rats

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Abstract

Physiologic elevation of insulin levels induces a significant increase in muscle adenosine triphosphate (ATP) synthesis rate in normal individuals, indicative of an appropriate acceleration in mitochondrial activity. However, the stimulatory effect of insulin is diminished in insulin-resistant patients. In the absence of similar data from preclinical models, the present study investigated the inhibitory effects of increased dietary fat intake on insulin-stimulated ATP synthesis rates in rats. After being placed on a high-fat diet for 8 weeks (n = 10), diet-induced obese male Sprague-Dawley rats were tested against age-matched control rats (n = 9) on a normal chow diet. Muscle ATP synthase flux rates were measured under anesthesia by in vivo ^{31}P saturation transfer both before and during a euglycemic-hyperinsulinemic clamp. The glucose infusion rates observed during the clamp revealed impaired peripheral insulin sensitivity in the high-fat–fed rats when compared with the age-matched control rats. Under baseline conditions (ie, low insulin), the muscle ATP synthesis rates of high-fat–fed rats were approximately 30% lower ($P < .05$) than those in chow-fed rats. Moreover, chow-fed animals showed a significant increase (25%, $P < .05$ vs basal) in muscle ATP synthesis activity upon insulin stimulation, whereas high-fat–fed animals displayed no substantial change. These data demonstrated for the first time in a preclinical model that the insulin challenge not only facilitates an improvement in the dynamic range of ATP turnover measurement by ^{31}P saturation transfer between normal and insulin-resistant rats, but also mimics challenge that is relevant for pharmacologic studies on antidiabetic drugs aimed at improving mitochondrial function.

1. Introduction

Therapeutic strategies aimed at increasing fatty acid oxidation in the muscle present promising targets for future treatments of type 2 diabetes mellitus (T2DM). Recent data obtained from the offspring of patients with T2DM suggest that an inherited defect in mitochondrial activity, assessed through in vivo measurement of the adenosine triphosphate (ATP) synthesis rate, is associated with intramyocellular lipid (IMCL) accumulation and may underlie the development of insulin resistance in muscle [1,2]. Although the causative nature of this relationship has not yet been established, modulating circulating lipid levels, either acutely (eg, through lipid infusion in humans [3]) or chronically (eg, by a high-fat diet regimen [4]), has shown that increased free fatty acid levels yield similar results both in terms of depressed ATP synthesis rates and elevated IMCL contents. Recent data have also shown that physiologically raising insulin levels may induce an up to 90% increase in the muscle ATP synthesis rate of normal individuals [2,3], whereas this stimulatory effect of insulin was moderated in insulin-resistant patients [2].

In view of such results, it has been suggested that a therapy aimed at improving the functionality of the mitochondrial system would increase the uptake of glucose into the muscle (insulin response) [5]. The need for highly sensitive biomarkers addressing mitochondrial activity has therefore led to the hypothesis that the measurement of ATP production may be more appropriate under insulin challenge conditions. To our knowledge, no such data have been demonstrated in preclinical models. To this end, the objective of this study was to ascertain an in vivo relationship between mitochondrial function, IMCL, and insulin resistance in rat muscle upon insulin stimulation. Muscle mitochondrial activity was determined using

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^{31}P saturation transfer, as recently published [4], measured before and during the steady state of a euglycemic-hyperinsulinemic clamp in anesthetized normal and diet-induced obese (DIO) rats.

2. Methods

2.1. Animals

Adult (12-14 weeks old) male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed with a 12-hour light/dark cycle and had free access to study diet and water. All experimental procedures were carried out in compliance with the guidelines and with the approval of the Novartis Institutional Animal Care and Use Committee.

2.2. Determination of optimal insulin infusate concentration

For this preliminary study, fifteen 12-week–old rats on normal chow diet (5053, 13% calories from fat; PicoLab Diet 20, Richmond, IN) were clamped using different concentrations of insulin infusate to determine which infusion rate would yield the highest glucose infusion rate (GIR) to be used in later in vivo metabolic studies. Animals were arbitrarily divided into 5 dose groups (2, 4, 8, 16, and 32 mU kg^{-1} min^{-1} insulin; Eli Lilly, Indianapolis, IN; n = 3 per group), and 4 to 6 rats were clamped each day over a 3-day period. Rats were fasted at 5:00 PM for the following day's study. On the day of the clamp, animals were anesthetized with 1% to 2% isoflurane (Baxter Healthcare, Deerfield, IL) for the cannulation of the jugular vein and carotid artery and maintained under anesthesia for the remainder of the study. Body temperature was regulated using heating pads, and respiration was visually monitored throughout the experiment. For the first 10 minutes of the insulin infusion, a bolus insulin infusion was given at 32 mU kg^{-1} min^{-1}, twice the constant infusion rate used for the remainder of the experiment. Blood glucose was sampled every 5 minutes, and plasma glucose was clamped at 140 mg/dL. At the end of 90 minutes, the rat was euthanized and plasma samples were processed.

2.3. Determination of muscle ATP synthesis rate and IMCL content

2.3.1. Animal setup

Measurements were carried out in 12- to 14-week–old rats fed either normal chow diet (n = 9) or fat-enriched diet (DIO; D12492i, 60% kcal; Research Diets, NJ; n = 10) from 4 weeks of age. As described in the preliminary study, rats were fasted at 5:00 PM the evening before the experiment; and dual cannulation was performed an hour before imaging. After surgery, the rat was laid prone on a supportive bed for imaging; and the cannula to the jugular vein was connected to 2 pumps, one containing 30% glucose and the other with 800 mU/mL insulin (Eli Lilly) in 0.1% bovine serum albumin in 154 mmol/l NaCl with 20 U/mL heparin, fixed beyond the magnetic field range. The rat was placed at the magnet isocenter and not moved until completion of the experiment. Basal ATP synthesis and the longitudinal (spin-lattice) relaxation time, T_1, for muscle inorganic phosphate (Pi) were measured by ^{31}P magnetic resonance spectroscopy (MRS) as described below. After the baseline measurements, the pumps were activated; and for the first 10 minutes (0-10 minutes), a bolus insulin infusion corresponding to 32 mU kg^{-1} min^{-1} was administered. At 10 minutes, the insulin infusion was reset to 16 mU kg^{-1} min^{-1}, as determined from the preliminary study described above. Blood drawn from the cannulated artery at baseline and then at 5-minute intervals during the infusion was used to monitor blood glucose levels, and the GIR was adjusted to achieve a steady-state blood glucose of 140 mg/dL. While reaching the steady state, IMCL content was measured by localized ^{1}H-MRS. Once the steady state was reached, ATP synthesis rates were again measured by ^{31}P-MRS. After the 90 minutes of euglycemic-hyperinsulinemic clamp, the experiment was stopped and the animal was euthanized.

2.3.2. In vivo MRS

All in vivo magnetic resonance (MR) measurements were performed on a Bruker Avance 7.0 T/30-cm wide-bore instrument (Bruker Medical, Billerica, MA) equipped with a 20-cm internal diameter actively shielded gradient insert. To collect signal from the lower leg of the rat, both ^{1}H and ^{31}P-MRS were performed using a dual-frequency ^{1}H/^{31}P 2.5-cm surface coil working in a transmitter/receiver mode and tuned to 300.31 (^{1}H) and 121.57 (^{31}P) MHz. The MRS data were obtained under 1% to 2% isoflurane anesthesia with constant monitoring of respiration and body temperature (SA Instruments, Stony Brook, NY). On average, total scanning time did not exceed 2.5 hours per animal.

Measurement of the ATP synthesis rate was systematically combined with the quantification of IMCL levels and determination of T_1 for muscle Pi before and during the clamp. After cannulation and pump line connection, the rat leg was carefully secured to the surface coil such that signal was generated mostly from the tibialis anterior (TA) between the knee and ankle. Orthogonal scout images were acquired using a fast imaging with steady-state precession sequence (echo time of 1.82 milliseconds , repetition time of 3.64 milliseconds, slice thickness of 2 mm, field of view of 35 × 35 mm, 8 averages) to confirm proper positioning. The MRS acquisition and subsequent calculations were carried out as described by Laurent et al [4]. A summary of the methods is presented below.

For the ATP synthesis rate determination, 2 spectra were acquired for each saturation transfer experiment, one with and one without (control spectrum) steady-state saturation of the ATPγ peak [6]. Each acquisition consisted of 128 averaged scans, each with a repetition time of 6 seconds, leading to a total acquisition time of 13 minutes per spectrum.

The observed spin-lattice relaxation time (T_{1obs}) of Pi was determined under basal conditions. As previously published [4], 6 inversion delays (ie, TI) were set for the accurate determination of T_{1obs}. Each inversion recovery spectrum was constituted of 32 averages, leading to a total experimental time of approximately 20 minutes. To verify that hyperinsulinemia had minimal influence on longitudinal relaxation, T_{1obs} was measured both prior and during the insulin infusion in a subset of animals (n = 6). Obtained results showed that insulin has no significant effect on the T_{1obs} of Pi (prior: 2.47 ± 0.10 seconds vs during: 2.34 ± 0.08 seconds, not significant [NS]).

After the baseline acquisition, basal blood glucose was measured and the clamp was initiated. While reaching the steady state of clamp, a 2 × 2 × 2-mm^3 volume of interest was carefully positioned in the scout images to be used to measure IMCL content in the left TA muscle, avoiding blood vessels and gross adipose tissue deposits [4,7,8]. Localized shimming was performed on water signal to achieve typical line widths of approximately 13 Hz. The localized ^{1}H-MR spectrum was then obtained using a point-resolved spectroscopy sequence (echo time of 20 milliseconds, repetition time of 2 seconds, 4096 data points over 8 kHz of spectral width, 256 scans) with chemical shift selective water suppression. As the steady state of clamp was reached, baseline shim and frequency settings were reinstated; and ATP synthesis rates were once again measured with 2 unsaturated followed by 2 saturated ATPγ acquisitions (total acquisition time of 55 minutes).

2.3.3. MRS data analysis

All ^{31}P spectra were processed using XWIN NMR Suite version 3.2 (Bruker Biospin, Karlsruhe, Germany). After applying gaussian apodization (20-Hz line broadening) and phase and baseline correction, peak areas were assessed for signals from Pi (4.9 ppm), creatine phosphate (0 ppm), and all 3 ATPs (γ-ATP: −2.4, α-ATP: −7.4, and β-ATP: −15.9 ppm). To gain sensitivity in the saturation transfer experiment, the saturation-induced change in Pi peak area (ie, ΔPi) was assessed after subtracting the γ-ATP saturated spectrum from the unsaturated control spectrum. The rest of the calculation for the determination of the ATP synthesis rate was performed as described earlier [4]. The IMCL spectra were processed using the Nuts-PPC software package (Acorn NMR, Fremont, CA). Once spectra were line broadened, phased, and baseline corrected, peak areas for total creatine (tCr: 3.02 ppm), extramyocellular lipids (methylene peak at 1.5 ppm), and IMCL (methylene peak at 1.3 ppm) were determined using a line-fitting procedure [4,7,8]. The IMCL content

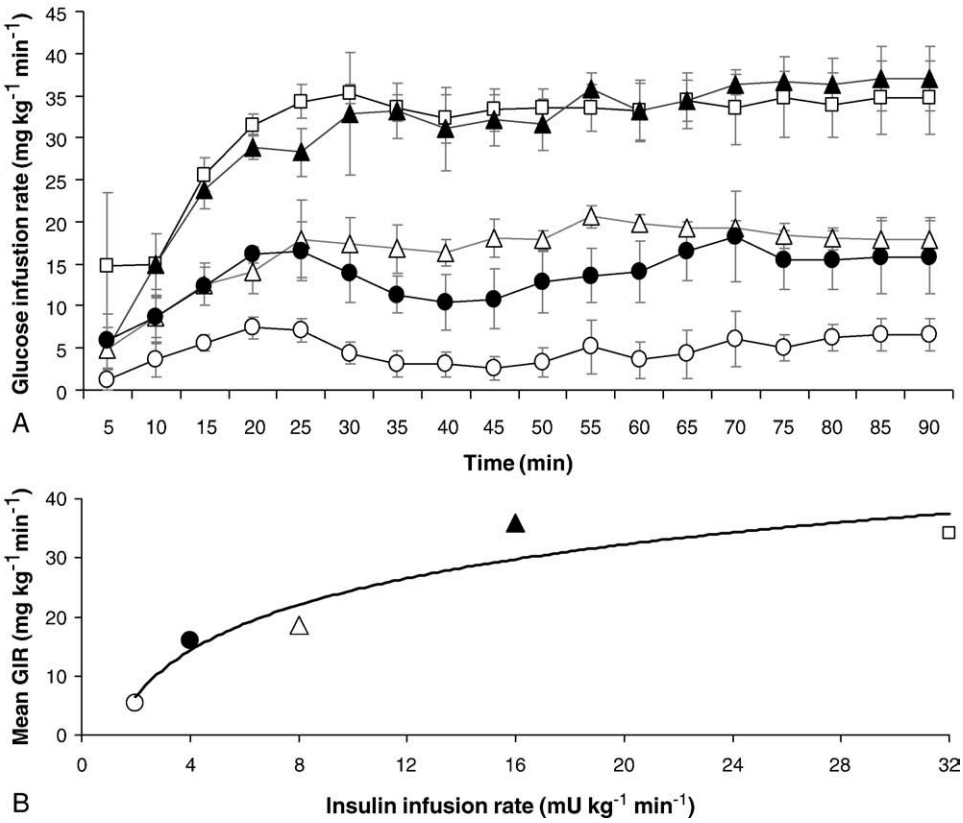

Fig. 1. A, Glucose infusion rate measured every 5 minutes during 90-minute euglycemic-hyperinsulinemic clamp. Insulin was infused at a constant rate of 20 μL kg^{-1} min^{-1} at 5 concentrations (2 mU [open circles], 4 mU [closed circles], 8 mU [open triangles], 16 mU [closed triangles], and 32 mU [open squares]; n = 3 per group). B, Logarithmic relationship (r = 0.95) between the mean GIR calculated between 60 and 90 minutes of the euglycemic-hyperinsulinemic clamp per group at each insulin concentration.

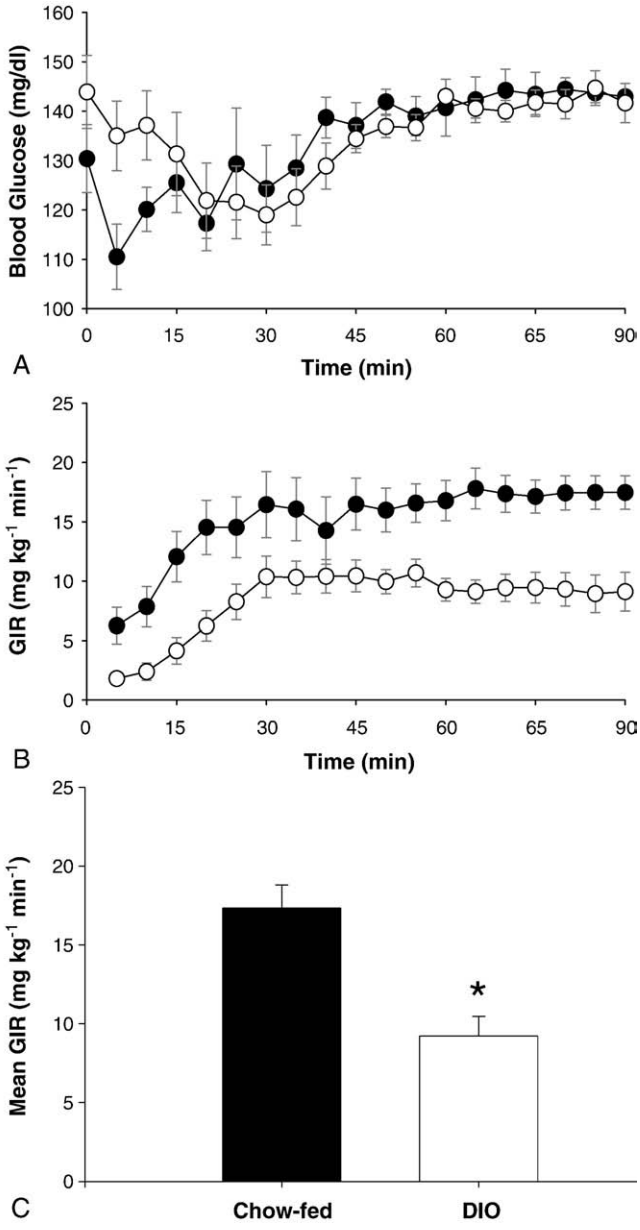

Fig. 2. Blood glucose (A) and GIR (B) measured every 5 minutes during 90-minute euglycemic-hyperinsulinemic clamp with 16 mU kg^{-1} min^{-1} insulin infusion. Higher GIRs were measured in chow-fed rats ($P < .05$, 2-way repeated-measures analysis of variance vs DIO rats). C, Mean GIR calculated between 60 and 90 minutes of clamp. Mean GIR was reduced by 45% to 50% (*$P < .05$) in DIO (open circles, white bar) vs chow-fed controls (closed circles, black bar).

was then expressed as a percentage of tCr content, the internal reference, as previously described [9].

2.3.4. Statistical analysis

Intergroup and intraanimal pairwise comparisons were made by unpaired and paired Student *t* tests, respectively. Where appropriate, correlations were assessed by linear and logarithmic regression analysis. To determine intergroup significance of longitudinal data, a 2-way repeated-measures analysis of variance was applied. Data are presented as means ± SEM, and *P* less than .05 was considered statistically significant.

3. Results

3.1. Determination of the optimal insulin infusate concentration

To select an appropriate insulin infusion rate, 5 concentrations of insulin infusate were tested on normal 12-week–old rats anesthetized with isoflurane. The data, presented in Fig. 1, showed that, at concentrations exceeding 16 mU kg^{-1} min^{-1}, GIR reached maximal values. Consequently, because the 16-mU kg^{-1} min^{-1} group gave the best insulin sensitivity–related response, it was used in the latter metabolic study.

3.2. In vivo MRS results

Basal glucose levels were 10% higher (NS, vs chow) in DIO rats. During the hyperinsulinemic-euglycemic clamp,

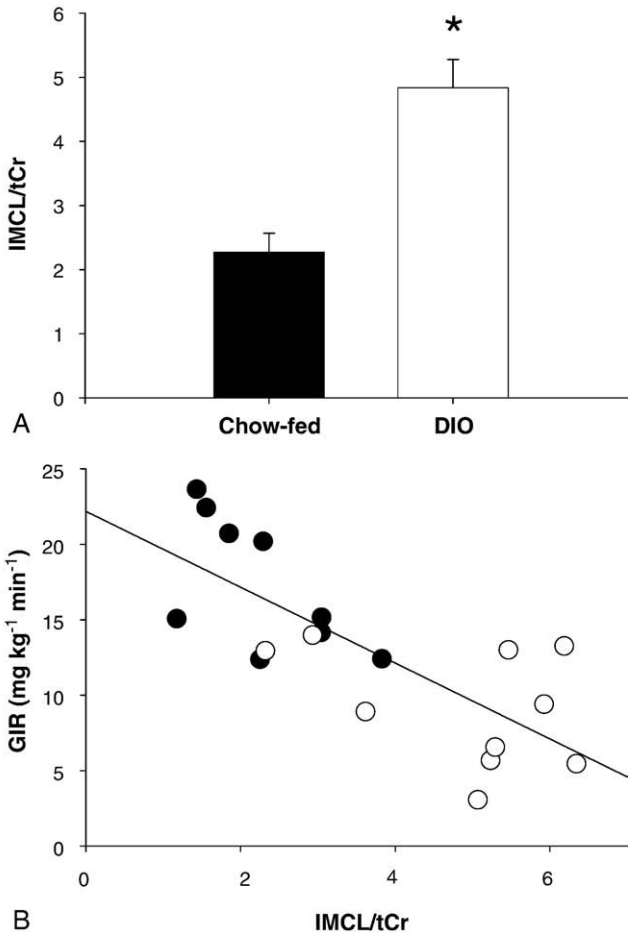

Fig. 3. A, Intramyocellular lipid to tCr ratio of the TA muscle by in vivo localized ^{1}H-MRS. Ten weeks on high-fat diet induced a 2-fold increase in IMCL (*$P < .05$) vs chow-fed controls. B, Negative correlation (all data: $r = 0.76$, $P < .001$) observed between GIR values and IMCL levels. Chow-fed (black bar, closed circles) and DIO rats (white bar, open circles).

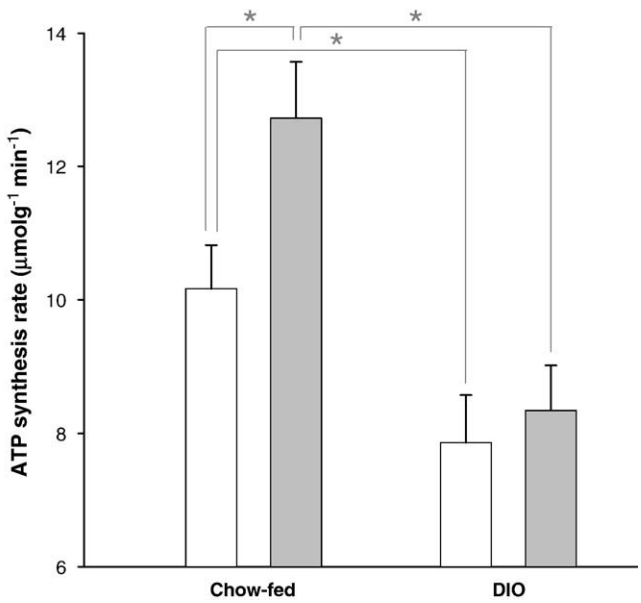

Fig. 4. Adenosine triphosphate synthesis rates measured in chow-fed (n = 9) and DIO rats (n = 10) before (white bars) and during (gray bars) the euglycemic-hyperinsulinemic clamp period. *$P < .05$.

animals reached a steady GIR after approximately 30 minutes (Fig. 2B); and GIR variability did not exceed 2% over the final 30 minutes of the clamp. The GIR results suggested that DIO rats were more insulin resistant than age-matched chow-fed rats (GIR 45%-50% less in DIO rats, $P < .05$) (Fig. 2C). The DIO rats showed significantly elevated levels of IMCL/tCr (DIO: 4.84 ± 0.44 vs chow: 2.54 ± 0.27, $P < .05$) (Fig. 3A), also supporting impairment of peripheral insulin sensitivity in the DIO rat model. The IMCL contents displayed a significant correlation with observed GIR ($r = 0.77$, $P < .05$) (Fig. 3B). Under basal conditions, ATP synthesis rates measured in chow-fed rats were 20% higher ($P < .05$) than those in DIO rats (Fig. 4) and, collectively, were significantly correlated with GIR values ($r = 0.50$ $P < .05$) (Fig. 5A). Upon insulin stimulation, chow-fed animals showed a significant increase (25%, $P < .05$) in ATP production, whereas age-matched DIO rats showed no substantial change (6%, NS) (Fig. 4). As a result, the initial dynamic range that was measured between chow-fed and DIO rats under basal conditions (ie, ~2.2 μmol g^{-1} min^{-1}) doubled (ie, ~4.4 μmol g^{-1} min^{-1}) in response to insulin stimulation ($P < .05$, basal vs clamp values). Finally, the correlation between GIR and ATP synthesis rates appeared to be stronger upon insulin stimulation ($r = 0.72$, $P < .005$) than under basal conditions (Fig. 5B), although the difference between the 2 correlation factors was not significant [10].

4. Discussion

Given the stimulatory role of insulin on muscle mitochondrial function [11], the primary purpose of this work was to assess whether insulin, in an anesthetized rat, induces an increase in muscle ATP production similar to the observation made in humans [12]. The existence of a reduced ATP synthase flux (~30%) in first-degree relatives of T2DM patients led the authors of the above-mentioned study to suggest an impairment of mitochondrial function as being a central factor to muscle insulin resistance and T2DM. In a different scenario, the potential activating role of insulin on the ATP synthesis rate was supported by an approximately 25% decrease (ie, approximately −1.8 μmol min^{-1} g^{-1}) in basal mitochondrial ATP production, determined in vitro from biopsies of muscle at rest, observed in patients with type 1 diabetes mellitus deprived of their normal regimen of long-acting insulin [13]. Likewise, although insulin stimulates ATP production in normal subjects, this response is blunted both in patients with T2DM [14] and in nonobese offspring of T2DM patients [2]. Although increased lipid availability has long been associated with impaired insulin

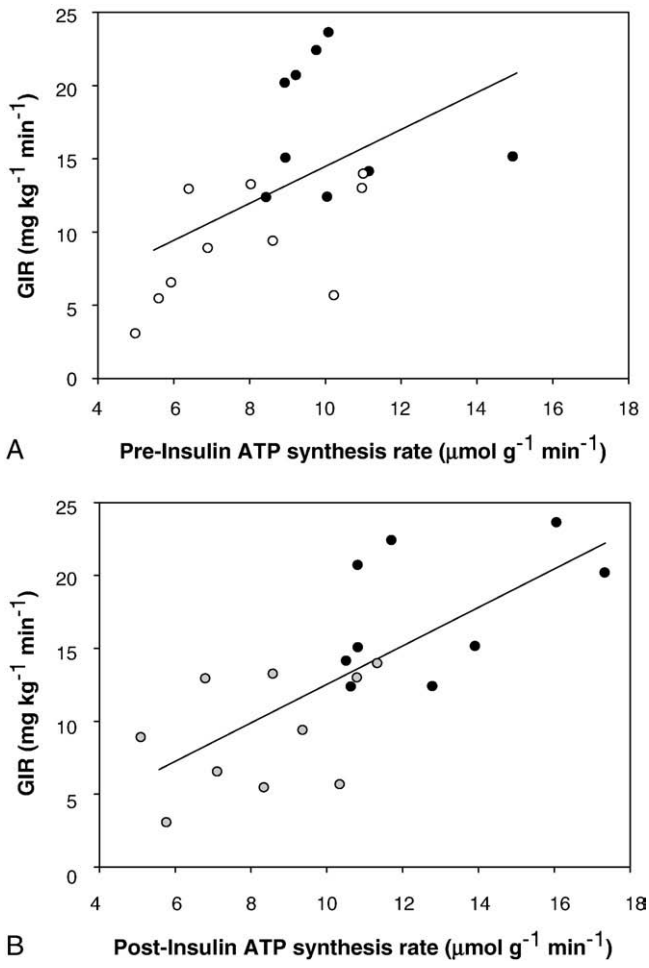

Fig. 5. Correlations between GIR and ATP synthesis rates both before (A) and during (B) the euglycemic-hyperinsulinemic clamp. The correlation coefficient appears improved (basal $r = 0.50$, whereas $r = 0.72$ with insulin stimulation) during the clamp steady state (NS). Chow-fed (closed circles) and DIO rats (open circles, pre–insulin challenge; gray circles, post–insulin challenge).

signaling [15,16], it has also been suggested that a reduction in peroxisome proliferator–activated receptor γ coactivator 1α, which is involved in mitochondrial biogenesis [17], may result from persistent consumption of excess dietary fat [18]. In this context, the present study investigated mitochondrial function in the DIO rat model with the assumption that persistent exposure to a high-fat diet would impair muscle mitochondrial function, perhaps through a mechanism involving lipid peroxidation [19,20].

Because mitochondrial ATP production is coupled to substrate utilization via a stoichiometric relationship [21], reduced ATP synthesis rates, as observed in the DIO rat model, are likely indicative of diminished mitochondrial activity [22]. This is assuming that glycolytic contribution to ATP synthesis (through, for example, glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions) is negligible in skeletal muscles, unlike in the heart, as the Pi $\rightarrow$ ATP flux is predominantly due to mitochondrial F_1F_0 ATPase activity [23]. However, the extent to which decreased mitochondrial density [24] or lessened efficiency of individual mitochondria [25] contributes to this effect is still debated. Because our experimental conditions involved an acute insulin response (not exceeding 90 minutes of insulin infusion), drastic changes in mitochondrial density would be unexpected, leading to the assumption that the insulin-induced response is a consequence of enhanced organelle activity over mitochondrial biogenesis. This would agree well with data from a comprehensive clinical study that demonstrated a specific augmentation in mitochondrial activity markers under clamp conditions [11]. Authors of this study reported an approximately 170% increase in cytochrome *c* oxidase messenger RNA transcript levels translating to a 20% to 25% increase in mitochondrial protein synthesis, which was well correlated with the 30% to 40% increase in ATP production measured. Although our study presented similar changes in ATP synthesis, it is important to note that our model used extraphysiologic doses of insulin (16 mU kg^{-1} min^{-1}) that were an order of magnitude higher than those used clinically (high dose: 1.5 mU $kg^{-1}_{lean\ mass}$ min^{-1} [11]). This discrepancy, which could result from inherent differences in the method (in vivo vs in vitro, species distinctions, or the use of anesthesia), requires additional investigation.

Furthermore, to support the ATP synthesis rate as a biomarker of muscle mitochondrial activity, the sensitivity and dynamic range of this readout may be explored in greater detail. Specifically, a study of the dose-response effect, while demonstrating the direct insulin dependence of ATP production, would also help validate the use of the high insulin infusion rate (16 mU kg^{-1} min^{-1}) for maximal impact on ATP flux.

Our previous finding has shown that persistent exposure to excess dietary fat decreases muscle ATP synthesis by up to 30% in an anesthetized rat [4]. The present study demonstrated that, with insulin stimulation, glucose uptake was associated with increased mitochondrial activity; and this response was "blunted" in the DIO rat, a model commonly used for peripheral insulin resistance. These data emulate findings shown clinically in insulin-resistant patients [2]. These results also confirm the importance of the insulin challenge for the study of mitochondrial function in a preclinical model, as it improves both the dynamic range and the sensitivity of the ATP readout compared with the basal fasting condition. The ATP synthase flux rate was reduced 20% in response to long-term feeding with lipid-rich diet under the normal anesthetized state. Although this decrease was significant, expanding the range between normal and DIO rats may allow more precise characterization of the state of insulin resistance. Stimulating the mitochondrial system with a steady insulin infusion enhanced the difference between normal and DIO rats to 35%. By nearly doubling the dynamic range, the sensitivity by which this preclinical assay can determine drug efficacy via a mitochondrial mechanism of action would also be improved.

In summary, our data not only confirm the degree of parallelism between the DIO rat model and the human condition of insulin resistance, but also establish the insulin-stimulated ^{31}P saturation transfer methodology as a fully back-translatable approach for the study of mitochondrial activity in skeletal muscles. Because skeletal muscle accounts for approximately 80% of glucose disposal, this noninvasive insulin-stimulated readout may be particularly useful in clinical and preclinical trials to determine the efficacy of new antidiabetic drugs.

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