A discordance in rosiglitazone mediated insulin sensitization and skeletal muscle mitochondrial content/activity in Type 2 diabetes mellitus

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Pagel-Langenickel I, Schwartz DR, Arena RA, Minerbi DC, Johnson DT, Waclawiw MA, Cannon RO 3rd, Balaban RS, Tripodi DJ, Sack MN. A discordance in rosiglitazone mediated insulin sensitization and skeletal muscle mitochondrial content/activity in Type 2 diabetes mellitus. Am J Physiol Heart Circ Physiol 293: H2659–H2666, 2007. First published September 21, 2007; doi:10.1152/ajpheart.00782.2007.—Skeletal muscle mitochondrial dysfunction is hypothesized to contribute to the pathophysiology of insulin resistance and Type 2 diabetes. Whether thiazolidinedione therapy enhances skeletal muscle mitochondrial function as a component of its insulin-sensitizing effect is unknown. To test this, we evaluated skeletal muscle mitochondria and exercise capacity in Type 2 diabetic subjects with otherwise normal cardiopulmonary function in response to rosiglitazone therapy. Twenty-three subjects were treated for 12 wk and underwent pre- and post-therapy metabolic stress testing and skeletal muscle biopsies. Rosiglitazone significantly ameliorated fasting glucose, insulin, and free fatty acid levels but did not augment the subjects’ maximal oxygen consumption (VO₂max) or their skeletal muscle mitochondrial copy number. The baseline VO₂max correlated strongly with muscle mitochondrial copy number (r = 0.56, P = 0.018, n = 17) and inversely with the duration of diabetes (r = −0.67, P = 0.004, n = 23). Despite the global lack of effect of rosiglitazone-mediated insulin sensitization on skeletal muscle mitochondria, subjects with the most preserved functional capacity demonstrated some plasticity in their mitochondria biology as evidenced by an upregulation of electron transfer chain proteins and in citrate synthase activity. This study demonstrates that the augmentation of skeletal muscle mitochondrial electron transfer chain content and/or bioenergetics is not a prerequisite for rosiglitazone-mediated improved insulin sensitivity. Moreover, in diabetic subjects, VO₂max reflects the duration of diabetes and skeletal muscle mitochondrial content. It remains to be determined whether longer-term insulin sensitization therapy with rosiglitazone will augment skeletal muscle mitochondrial bioenergetics in those diabetic subjects with relatively preserved basal aerobic capacity.

exercise capacity; citrate synthase activity; peroxisome proliferator-activated receptor-γ agonist

DIMINISHED SKELETAL MUSCLE glucose uptake and attenuated skeletal muscle insulin sensitivity are important precursors to the development of Type 2 diabetes mellitus (19). Skeletal muscle of insulin-resistant subjects has diminished mitochondrial density (24) and reduced mitochondrial oxidative phosphorylation (27). Diabetic individuals also show coordinate downregulation of genes encoding the oxidative phosphorylation enzymes (22) and diminished skeletal muscle mitochondrial respiration (23), and evidence of reduced skeletal muscle bioenergetic capacity as illustrated by slower recovery of skeletal muscle high-energy phosphate stores in response to exercise compared with nondiabetic controls (32). The proposed mechanism whereby disruption in skeletal muscle mitochondria exacerbates insulin resistance has been recently reviewed and suggests that the resultant reduction in fatty acid β-oxidation increases intracellular fatty acyl-CoA and diacylglycerol, which phosphorylates and inactivates insulin signal transduction intermediates via protein kinase C signaling (18). Collectively, these data suggest that disruption of the skeletal muscle mitochondrial biogenesis regulatory program and bioenergetics are integral to insulin resistance in skeletal muscle and raise the question as to whether these perturbations exacerbate the progressive pathophysiology of Type 2 diabetes mellitus. Interestingly, the thiazolidinedione insulin sensitizers delay the onset of Type 2 diabetes (7) and have been shown to restore mitochondrial respiratory function and biogenesis in adipose tissue (4, 39). In light of the importance of skeletal muscle insulin sensitivity in the development of insulin resistance and diabetes, we evaluated whether the augmentation of mitochondrial bioenergetics in skeletal muscle may be an important component in rosiglitazone-mediated improvement in insulin sensitivity and glycemic control. Furthermore, if this biology is operational, we would expect an improvement in exercise capacity in parallel with an improvement in skeletal muscle mitochondrial function.

To establish the effect of thiazolidinedione therapy on skeletal muscle biology, we performed studies on skeletal muscle biopsies and compared these with metabolic stress tests in diabetic subjects in response to 12 wk of rosiglitazone therapy. Although improvement in glycemic control was a uniform
finding, there was no augmentation in the study subjects' aerobic capacity. On direct assessment of mitochondrial proteins, an induction of mitochondrial oxidative metabolic protein content and activity was evident in the subset of study subjects with most robust baseline aerobic capacity. Furthermore, baseline $\dot{V}O_{2\text{max}}$ was shown to be a strong indicator of the duration of diabetes and of underlying skeletal muscle mitochondrial genomic content. Taken together, these data show that the improvement in skeletal muscle mitochondrial function is not mechanistically linked to rosiglitazone-mediated insulin sensitization. A hypothesis generated by this study is that the capacity for skeletal muscle mitochondrial bioenergetic augmentation in diabetes subjects may be dependent on the duration of diabetes and on a subject's baseline maximal exercise capacity.

METHODS

Study design. This was a prospective open-labeled study to evaluate the skeletal muscle mitochondrial response to the addition of rosiglitazone to the therapeutic regimen of diabetic subjects naïve to thiazolidinedione therapy. Subjects were excluded from participation if they had abnormal cardiopulmonary function or an inability to exercise on a treadmill. Subjects were maintained on their prestudy therapy, and rosiglitazone was introduced and titrated up from 2 mg daily to a maximum of 4 mg two times daily, or to the highest dose tolerated, over a 6-wk period. Therapy was continued for an additional 6 wk. All subjects had an initial visit with the dietician, but no further attempt was made to modify their exercise regimen or dietary habits, since the objective of this study was to assess the exclusive effect of rosiglitazone on maximal effort tolerance and on skeletal muscle biology. This study was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute, and all subjects provided informed written consent.

Clinical evaluation. Subjects were seen biweekly to adjust dosing and then monthly once they were on their maximal tolerated dose. Additional baseline studies included metabolic stress testing and gastrocnemius muscle biopsy that was performed 5 days after the maximal stress test. Baseline and final insulin sensitivity was calculated using the QuickI formula, which incorporates fasting insulin and glucose values and correlates with the oral glucose tolerance test (29). Additional baseline studies included metabolic stress testing and a gastrocnemius muscle biopsy that was performed >5 days after the maximal stress test. Baseline and final insulin sensitivity was calculated using the QuickI formula, which incorporates fasting insulin and glucose values and correlates with the oral glucose tolerance test (29).

Finally, brachial artery reactivity was assessed at baseline and at 12 wk to determine effects of the thiazolidinediones on endothelial nitric oxide bioactivity. Endothelium-dependent flow-mediated vasodilation was assessed by Doppler imaging, as previously reported (9).

Skeletal muscle analyses. Symptom-limited cardiopulmonary treadmill exercise testing was performed 1) at baseline and post study. Ventilatory expired gas analysis was collected using a metabolic cart (Senoromedics; Viasys Healthcare, Conshohoken, PA). Oxygen uptake and carbon dioxide output were acquired, and the maximal oxygen consumption ($\dot{V}O_{2\text{max}}$) and peak respiratory exchange ratio (RER) were expressed as the highest 10-s averaged samples obtained during the last stage of the exercise test.

Genomic DNA was extracted from a random selection of 17 subjects to use for analysis of mitochondrial copy number. Mitochondrial DNA copy number was assessed as described previously (15). In brief, DNA was collected from whole tissue lysates, and quantitative real-time PCR was performed for mitochondrial-encoded cytochrome c oxidase (COX) subunit III and for nuclear-encoded 18S using SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and the MJ Research DNA Engine Opticon 2 fluorescence detection system. The ratio of COXIII DNA copies to 18S DNA represents the relative mitochondrial copy number.

Since proteomic analysis is a nonbiased technique to identify regulation at the protein expression level, a random sample of six subjects to use for analysis of mitochondrial copy number. Mitochondrial-encoded COXII complexes (Mitosciences, Eugene, OR). Bands were visualized using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and the SuperSignal West pico chemiluminescence substrate kit (Pierce Biotechnology, Rockford, IL).

Citrate synthase activity was measured in all skeletal muscle samples ($n = 23$) using 10–25 µg of protein from whole tissue skeletal muscle. Activity level, expressed as micromoles per gram total protein per minute, was measured spectrophotometrically using a commercially available kit (Sigma).

Statistical analysis. Differences between subjects were evaluated for significance using the paired (pre and post rosiglitazone) and unpaired (between subgroups) two-tailed Student's $t$-test. Regression analysis was used to assess relationships between $\dot{V}O_{2\text{max}}$ and percent change in citrate synthase activity, duration of diabetes, mitochondrial copy number, and endothelial function. Since citrate synthase activity reflects mitochondrial oxidative metabolism, all tissue samples were used to measure this activity. The regression analysis comparing $\dot{V}O_{2\text{max}}$ with the change in citrate synthase activity in response to rosiglitazone therapy was considered the primary comparison. The limited biopsy material was then randomly designated for proteomic analysis or for the determination of mitochondrial copy number and Western blot analyses. In light of the small sample sizes, these subgroup analyses are viewed as exploratory data to determine whether these biological readouts support the primary comparison. Receiver operating characteristic curve analysis determined the optimal $\dot{V}O_{2\text{max}}$ threshold at baseline to predict improvement in citrate synthase activity. A two-sided Fisher exact test was used to determine the relative effects of the use of other antidiabetic drugs in subgroup analysis. All data are expressed as means ± SE. $P < 0.05$ was considered statistically significant in all analyses.

RESULTS

Clinical response. Twenty-five individuals entered the study (age 60 ± 2 yr, 19 males and 6 females). Two subjects did not complete the study because one individual declined the second biopsy and one developed cellulitis following the first biopsy and we elected not to repeat the procedure. Overall, the therapy was well tolerated, with 86% of subjects reaching the maximal dose, the average dose being 7.5 ± 1 mg daily. The baseline body mass index of subjects enrolled in the study was 31 ± 0.9 kg/m², and the most common side effect was further weight gain, with 31% of study subjects gaining more than 2 kg over the 12-wk study. The efficacy of rosiglitazone is shown in Table 1. These changes are consistent with previous studies showing significant reductions in fasting glucose, fasting insulin, in free fatty acids, and in the highly sensitive C-reactive protein. Interestingly, there was no change in the glycosylated hemoglobin levels, which may reflect the good control already evident in these subjects with a baseline HbA1c of 6.5 ± 0.16%. Despite the improvement in serological parameters, there was no significant change in $\dot{V}O_{2\text{max}}$ (from 24.6 ± 1.3 to 24.7 ± 1.2 ml·kg⁻¹·min⁻¹, $P = 0.89$) after 12 wk of rosiglitazone. Furthermore, peak RER values were >1.10 during both...
V̇O₂max could be used to study skeletal muscle mitochondrial function, and the baseline and postintervention exercise tests, indicating that the subjects put forth a maximal, comparable effort (baseline 1.17 ± 0.08, postintervention 1.17 ± 0.09, P = 0.96).

Maximal exercise capacity and diabetes. To assess whether V̇O₂max could be used to study skeletal muscle mitochondrial function, the relative mitochondrial to nuclear DNA copy number was determined in 17 randomly selected diabetic subjects. The mitochondrial genomic copies of COXIII correlated strongly with V̇O₂max (r = 0.56, P = 0.0184, n = 17; Fig. 1A). The association between the severity of diabetes with aerobic capacity was demonstrated by inverse correlations between baseline V̇O₂max and the duration of diabetes (r = −0.67, P = 0.004, n = 23; Fig. 1B). A correlation between insulin sensitivity and V̇O₂max did not reach statistical significance (r = 0.30, P = 0.069), and age did not correlate with V̇O₂max.

Electron transfer chain protein modulation in response to rosiglitazone. Since the V̇O₂max was not changed in the group as a whole, we employed unbiased proteomic analysis to determine whether regulatory changes were evident with direct assessment of mitochondrial oxidative phosphorylation proteins. A randomly chosen subset of six study subjects’ whole muscle samples were employed for this analysis. In duplicate samples, 305 peptide sequences were identified as high priority, defined as a high confidence that the peptide sequences are correct and that multiple peptide sequences of the same protein are obtained. Of these 305 high-priority sequences, 9 proteins were identified as involved in mitochondrial oxidative phosphorylation. In this small subject sample size, only the subject with the highest V̇O₂max displayed a coordinate induction of the oxidative phosphorylation proteins (Fig. 2). Albeit preliminary and limited in scope, this screening test suggested that skeletal muscle mitochondrial plasticity in response to insulin sensitization therapy may be achievable in those subjects with higher basal aerobic capacity. We then evaluated the change in citrate synthase activity, a measure of mitochondrial oxidative metabolism (36) and/or of mitochondrial content (12), in all 23 subjects compared with basal V̇O₂max. The change in citrate synthase activity was correlated with the baseline V̇O₂max (r = 0.54, P = 0.01). Because this correlation elicited greater significance employing an exponential fit (r = 0.47, P = 0.001), we calculated the threshold of V̇O₂max delineating an improvement, or not, in citrate synthase activity in response to rosiglitazone therapy. The threshold of ≥28.1 ml·kg⁻¹·min⁻¹ provided a sensitivity of 70% and a specificity of 92% between responders and nonresponders.

Table 1. Effect of rosiglitazone on serum markers of insulin resistance and flow-mediated dilation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Final</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>130±3.5</td>
<td>112±2.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting insulin, µU/ml</td>
<td>23.5±4.5</td>
<td>17.3±3.5</td>
<td>0.015</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>0.30±0.01</td>
<td>0.32±0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FFA, µeq/ml</td>
<td>6.50±0.16</td>
<td>6.48±0.11</td>
<td>0.82</td>
</tr>
<tr>
<td>hsCRP, mg/dl</td>
<td>499±48</td>
<td>345±47</td>
<td>0.02</td>
</tr>
<tr>
<td>Flow-mediated dilation, %</td>
<td>0.40±0.09</td>
<td>0.18±0.03</td>
<td>0.02</td>
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</table>

Values are means ± SE. FFA, free fatty acid. hsCRP, highly sensitive C-reactive protein.

All subsequent subgroup analysis was performed comparing subjects with a basal V̇O₂max of <28.1 ml·kg⁻¹·min⁻¹ with those with values ≥28.1 ml·kg⁻¹·min⁻¹. The basal mitochondrial copy number was significantly higher in subjects with a V̇O₂max of ≥28.1 ml·kg⁻¹·min⁻¹ (Fig. 3A), and the duration of diabetes was significantly shorter in these subjects, with a mean duration of 30 ± 6.6 mo vs. 84 ± 11 mo (P = 0.0196) in subjects with reduced V̇O₂max and lower mitochondrial copy number (Fig. 3B). There was no difference in age between subjects with V̇O₂max of ≥28.1 ml·kg⁻¹·min⁻¹ (61.1 ± 3 vs. 58.1 ± 2.4 yr, respectively, P = 0.48; Fig. 3C). Interestingly, baseline citrate synthase activity did not correlate with V̇O₂max. The association between aerobic capacity and the upregulation in oxidative phosphorylation proteins in response to rosiglitazone in this subset of diabetic subjects was further supported by the parallel increase in citrate synthase activity by 53 ± 35.8% compared with −24 ± 10.6% (P = 0.03) in subjects with lower aerobic capacity and modest attenuation in oxida-
ROSIGLITAZONE AND SKELETAL MUSCLE MITOCHONDRIA

Because skeletal muscle is a predominant reservoir for postprandial glucose uptake (21) and skeletal muscle insulin resistance is proposed to be pivotal in the development of diabetes, the question arising is whether restoration of mitochondrial biology in skeletal muscle is central to the insulin-sensitizing effects of the thiazolidinediones. This question formed the central objective of this study.

Because VO$_2$max represents a composite measure of cardiac, pulmonary, skeletal muscle, and vascular function (37), we reasoned that the evaluation of the modulation in VO$_2$max in diabetic subjects with a normal cardiopulmonary profile could be employed as an indirect measure of skeletal muscle bioenergetic changes in response to rosiglitazone. Furthermore, the direct association of VO$_2$max with skeletal muscle bioenergetics is supported by the strong linear correlation between hyperinsulinemia-mediated skeletal muscle glucose uptake and VO$_2$max in insulin-resistant individuals (25). At the cellular level, this association is more directly linked to the mitochondria in that a relative reduction in type I oxidative fibers (16) and an increased ratio of glycolytic to oxidative metabolic enzymatic activity (19). This is hypothesized to result from, in part, reduced skeletal muscle mitochondrial oxidative capacity-evoked fatty acid metabolite accumulation with the subsequent disruption in insulin signal transduction (24). It has recently been demonstrated that skeletal muscle mitochondrial substrate oxidation is impaired in insulin-resistant offspring of Type 2 diabetic patients (2). Whether the augmentation of skeletal muscle mitochondrial function is indeed required to improve insulin sensitivity has not been established. The PPARγ agonist rosiglitazone has been shown to improve mitochondrial respiration in adipocytes of ob/ob mice (39) and to upregulate the adipocyte mitochondrial biogenesis regulatory program in two mouse models of obesity/diabetes (31).

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in V̇O₂max in response to rosiglitazone by Regensteiner and colleagues (30). The major difference between our study and the Regensteiner study is that they treated subjects for 4 mo vs. the 3 mo in our study. This modestly longer duration of therapy would support additional improvement with an extension in the duration of therapy. However, while our manuscript was under review, a study was published evaluating the effect of rosiglitazone on skeletal muscle mitochondria in high-fat-fed rats (14). In parallel with our study, Lessard et al. (14) demonstrated that a short duration of treatment did not augment skeletal muscle mitochondrial function but did ameliorate the adverse effects of the high-fat diet on adipose tissue and in the liver.

The more direct evaluation of skeletal muscle mitochondria included quantification of the relative change in skeletal muscle mitochondria genomic content, the change in activity of citrate synthase [a measure that reflects mitochondrial content (12) and/or oxidative activity (36)], and the steady-state protein levels of electron transfer proteins. The lack of change in mitochondrial genomic copy number is consistent with our V̇O₂max data. Because citrate synthase activity has been shown to represent oxidative metabolism in skeletal muscle, the lack of correlation between baseline citrate synthase activity and V̇O₂max is not readily interpretable. However, sedentary lifestyle, obesity, and insulin resistance all contribute to suppression of citrate synthase activity (8, 34), and because our

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

Fig. 3. Comparison of lower and higher baseline V̇O₂max with diabetic parameters. A: relative mitochondrial copy number contrasting diabetic subjects with a V̇O₂max below and above the threshold of 28.1 ml·kg⁻¹·min⁻¹. B: historical duration of diabetes compared with the same threshold of V̇O₂max. C: comparison of study subjects’ age in the V̇O₂max-delineated subgroups. D: change in citrate synthase activity in response to rosiglitazone therapy comparing subjects as delineated by their baseline V̇O₂max.
ROsiglitazone and Skeletal Muscle Mitochondria

Subjects have various combinations of these phenotypes, the number of subjects investigated may have been insufficient to clearly demonstrate a correlation between baseline citrate synthase activity and VO2max. Inversely, the potential to reverse the skeletal muscle mitochondrial defects in diabetes is evident as shown by the coordinate induction of citrate synthase activity and the upregulation of mitochondrial oxidative metabolic proteins exclusively in the subset of subjects with relatively preserved maximal aerobic capacity. On a cautionary note, these ameliorative changes were only present in a minority of the diabetic subjects studied. Hence, whether a mitochondrial genomic content threshold predicates the capacity to augment the diabetic subjects studied. Hence, whether a mitochondrial

change in citrate synthase activity in the individuals with increased basal VO2max.

Disruption of skeletal muscle mitochondrial function is evident early in the development of diabetes (24, 26–28) and may exacerbate insulin resistance via the accumulation of intracellular fatty acid metabolites (6, 18). Although it is likely secondary to insulin resistance, first-degree relatives of Type 2 diabetics show perturbations in insulin signaling and glucose transport preceding the dysregulation of mitochondrial biogenesis regulatory genes (11). In this study, the uniformly improved glycemic control and insulin sensitivity independent of skeletal muscle mitochondrial content/activity supports that, at least as an acute response, improvement in skeletal muscle mitochondrial biology is not a prerequisite for rosiglitazone-mediated glycemic control. This does not exclude the involvement of adipocyte mitochondrial biology or the possibility that more prolonged therapy may further enhance insulin sensitivity, in part by induction of skeletal muscle mitochondrial defects in diabetes.

Table 2. Effect of rosiglitazone on serum markers of insulin resistance and flow-mediated dilation depending on baseline VO2max

<table>
<thead>
<tr>
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<th>VO2max &lt;28.1 (n = 13)</th>
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<td></td>
<td>Baseline</td>
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<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>130±4.7</td>
<td>113±13</td>
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<tr>
<td>Fasting insulin, μU/ml</td>
<td>28.1±7.0</td>
<td>18.7±4.2</td>
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<td>Insulin sensitivity</td>
<td>0.29±0.01</td>
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</tr>
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<td>FFA, μeq/ml</td>
<td>527±69</td>
<td>388±76</td>
</tr>
<tr>
<td>hsCRP, mg/dl</td>
<td>0.40±0.06</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>Flow-mediated dilation, %</td>
<td>6.58±0.9</td>
<td>6.96±1.0</td>
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Values are means ± SE.
drial bioenergetics. In fact, the difference in plasticity seen in our study compared with the robust induction of mitochondrial biogenesis in adipocytes in response to rosiglitazone (31) may reflect the relative lower abundance of PPARγ in skeletal muscle compared with expression in adipose tissue (17, 35).

From a clinical perspective, we propose that these data support the determination of \( \text{VO}_{2\text{max}} \) in diabetic individuals with otherwise normal cardiopulmonary function as a functional test to differentiate those earlier and later in the course of the disease. Interestingly, the biological importance of the measurement of \( \text{VO}_{2\text{max}} \) in diabetic subjects has been further underscored by the recent observation that the maximal exercise capacity predicts mortality in Type 2 diabetes (20). In light of the accumulating evidence supporting interventions to delay the progression of diabetes (7, 13), the finding of a relatively maintained \( \text{VO}_{2\text{max}} \) may have added motivational benefit for these individuals identified as being in the early stages of this disease. Moreover, if skeletal muscle bioenergetic reversibility is dependent on early intervention in disease progression, this would support metabolic testing as a screening tool. Prior to this, however, a more comprehensive study in diabetic subjects would support metabolic testing as a screening tool. Prior to this, however, a more comprehensive study in diabetic subjects with otherwise normal cardiopulmonary function as a functional test to differentiate those earlier and later in the course of the disease would support metabolic testing as a screening tool.

Overall, this study illustrates new mechanistic insight into the temporal pathophysiology in skeletal muscle of diabetes with respect to mitochondrial plasticity and the effects of rosiglitazone. We conclude that the major finding in this study is that rosiglitazone augments insulin sensitivity independent of \( \text{VO}_{2\text{max}} \), demonstrating that the improvement in skeletal mitochondrial function is not required for rosiglitazone-mediated insulin sensitization. Furthermore, this study shows that the measurement of \( \text{VO}_{2\text{max}} \) in Type 2 diabetics can predict the extent and duration of their disease and reflects the underlying skeletal muscle mitochondrial content.

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