Restoring skeletal muscle insulin resistance and fatty acids oxidation by AICAR rescues proglycogen but not macroglycogen pools

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ABSTRACT

We examined in isolated soleus muscle (1) the effects of palmitate-induced insulin resistance on pro-and macro-glycogen pools; and (2) the responses of these pools to rescue of insulin resistance by AICAR treatment. Incubation of soleus muscle with palmitate (2mM) for ≤18 induced insulin resistance. Providing AICAR in the last 6h of incubation rescues insulin resistance. Changes in PG and MG pools, palmitate oxidation, and insulin-stimulated glucose transport were examined at 0, 6,12 and 18h.

Inducing insulin resistance by palmitate treatment for 18 h reduced PG pool (-62%), fatty acid oxidation (-50%), and insulin-stimulated glucose transport (-87%). However, no change was observed on MG pool. On the other hand, rectifying insulin resistance by AICAR treatment (12-18 h) fully rescued PG pool, palmitate oxidation, and insulin-stimulated glucose transport, but had no effect on MG pool.

In conclusion this study demonstrates for the first time that PG pool rather than MG pool is preferentially utilized during insulin resistance induced by prolonged incubation with palmitate.

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INTRODUCTION

The energy of the skeletal muscle is largely produced as a consequence of the oxidative metabolism of glucose and fatty acid[1]. Skeletal muscle is a principal site of insulin-stimulated glucose disposal, either for oxidation or for storage as glycogen[2-4]. Glycogen stores in skeletal muscle represent an important source of energy[5] and their breakdown is closely regulated in relation to increased energy demand. It is now clear that glycogen is not a uniform molecule, and it exists in two pools: proglycogen the dynamic intermediate form, and macroglycogen the inactive storage form[6-10]. Both proglycogen and macroglycogen are suitable substrates for energy production during skeletal muscle contraction[6,11], with macroglycogen mainly being utilized during aerobic exercise[6,12] while proglycogen mainly utilized during anaerobic exercise[6,9].

Skeletal muscles in addition to being a metabolic sink for circulating glucose, it also takes up significant quantities of circulating fatty acids, either for oxidation or for storage[1,14-16]. Therefore, skeletal muscle can metabolize both glucose and fatty acids, and it is characterized by its ability to readily switch between these fuel sources according to their availability[1,15,16]. However, in insulin resistance state like type 2 diabetes, the ca-

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pacity of skeletal muscle to take up and utilize either glucose or fatty acids as fuel and to switch between them is profoundly altered[16-20].

Recent works from our lab had shown that incubation (0-18h) of soleus muscle with palmitate (2mM) can readily induce insulin resistance characterized by marked reduction in insulin-stimulated glucose transport[17,18,21], and this effect was closely accompanied by a marked reduction in the rate of fatty acid oxidation[17,21]. In this model we have also been able to rapidly rescued palmitate-induced insulin resistance by a number of factors including; 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), leptin, and thujone[17,21]. These positive effects were also associated with improvements in fatty acid oxidation[17,21]. To the best of our knowledge, there is no information available about the effect of insulin resistance induced by palmitate treatment (0-18h) on proglycogen and macroglycogen pools. Similarly, the responses of these pools to rescue of insulin resistance by AICAR treatment have not been examined. Therefore, in the present study, we have used this model 1) to investigate possible effects of insulin resistance induced by prolonged palmitate treatment on proglycogen and macroglycogen pools and 2) to examine the responses of these pools to rescue of insulin resistance by AICAR treatment. For these purposes, we rapidly, i) induced insulin resistance with a high concentration of palmitate (2mM, 0-18h), and ii) subsequently, we attempted to rectify insulin resistance with AICAR treatment, while maintaining high concentrations of palmitate. We hypothesized that the proglycogen pool would be most affected by these changes in fatty acid and glucose metabolism, because this pool is known to be more dynamic, and more sensitive[5,6,9].

METHODS

Materials

Collagenase type II was purchased from Worthington (Lakewood, NJ). Insulin (Humulin-R) was purchased from Eli-Lilly (Toronto, Ontario). Penicillin and streptomycin were purchased from Invitrogen Corporation (Grand island, NY, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Animals

All experiments were approved by the Committee on Animal Care, at the University of Guelph. Male Sprague-Dawley rats (55-75g) were bred on sites and consumed normal laboratory chow and water ad libitum. For each experiment rats were anesthetized with Somnotol (6 mg·(100 g body weight)$^{-1}$, i.p.), and the soleus muscles were dissected.

Muscle incubation

Briefly, after a 30 min preincubation, soleus muscles (~20 mg), were incubated without (control) or with palmitate (2 mM) up to 18 hours. All incubations (0–18 h) were performed in 10 ml of warmed (30°C), pregassed (95% O2-5% CO2) Medium 199 containing 5 mM glucose supplemented with 4% bovine serum albumin V (BSA), penicillin (100 IU/ml), and streptomycin (0.1 mg/ml). Low concentrations of insulin (14.3 μU/ml) were also included, the low concentrations of insulin did not stimulate glucose transport (data not shown). Incubation vials were shaken at 110 cycles/min, and the gas phase and temperature were maintained at 95% O2-5% CO2 and 30°C, respectively. The incubation medium was replenished every 6 h. After 12 h incubation with palmitate, some muscles were incubated for an additional 6 h with AICAR (2mM).

Glucose transport

[^{3}H]-3-O-methyl-D-glucose (3-O-MG) transport was determined as we have previously described[17, 18]. Briefly, at the end of the incubation periods (0-18h), soleus muscles were incubated (30°C, 30 min, 95% O2-5% CO2) in 2ml of palmitate-free Krebs-Henseleit buffer [8 mM glucose, 32 mM mannitol, and 0.1% BSA with (20 mU/ml) or without insulin]. Subsequently, muscles were washed (2 *(10 min, 30°C, glucose-free Krebs-Henseleit buffer, 40 mM mannitol, 0.1% BSA, with [20 mU/ ml] or without insulin)). Glucose transport was then determined in palmitate-free Krebs-Henseleit buffer (2 ml) supplemented with 0.5 μCi [^{3}H]-3-O-MG, 1.0 μCi [^{14}C]-mannitol, 32 mM 3-O-MG, 4 mM mannitol, 4 mM pyruvate, and 0.1% BSA, in the presence (20 mU/ml) or absence of insulin for 20 min, as previously reported. Thereafter, muscles were blot-

ted, weighed, and solubilized followed by scintillation counting of muscle extracts.

Palmitate oxidation

To determine the rate of FA oxidation, our previously described method was used[17,18]. Briefly, at the end of the incubation period (0-18h) muscles that were incubated with and without palmitate were transferred to glass vials containing 2 ml pregassed (95%O2-5%CO2) medium 199 supplemented with 4% BSA and palmitate (2mM, 0.5μCi/ml of [1-14C]-palmitate. Palmitate oxidation occurred at 30°C for 40 min and the $^{14}CO_2$ released was captured in a benzothium hydroxide trap (400 μl, 1.0 M). In addition, at the end of the 40-min incubation period, dissolved CO_2 was also captured in this manner by adding sulfuric acid (1.0 ml, 1 M) to a 1.0-ml aliquot of the incubating medium. Finally, water-soluble ^{14}C-labeled intermediates were extracted from muscles homogenized after their incubation. After scintillation counting, the palmitate oxidation rate was determined by summing the three sources of [^{14}C] palmitate[17,18,21]

Glycogen extraction and determination

Extraction and determination of muscle macroglycogen (MG) and proglycogen (PG) were performed as we described previously[22]. For muscle glycogen extraction the digestion began by adding 200μL of ice-cooled 1.5 mol/L perchloric acid (PCA) to 2-3 mg of freeze-dried muscle sample in 5ml labeled pyrex tubes submerged in ice bath. The extraction continued on ice for 20min. During that period of time, muscle was pressed against the tube by plastic rod to ensure complete extraction of muscle glycogen. Tissue debris was pelleted by centrifugation at 1600g for 15 min at 4°C, after which 100μL of the supernatant fraction was collected and used for MG determination. The remaining pellet was kept and used for PG determination.

1mL of 1mol/L HCl was added to the PG and MG fractions, and the PG fraction was thoroughly agitated with a plastic rod to ensure that all muscle debris was exposed to PCA. Thereafter, the samples were placed in sealed glass-stoppered tube and incubated in a water bath for 2h at 100 °C. The samples were then neutralized by the addition of 700μL of 2 mol/L Tris base. After vortexing and centrifugating the samples at 1500g

for 5min at 4°C, the supernatant was removed, transferred to labeled Eppendorf tubes, and stored at -80°C for later analysis of glucosyl units. PG and MG portions were determined enzymatically using a hexokinase reaction. Standard calculations were performed to determine the concentration of glucosyl units in muscle base on the standard curve. Total glycogen was determined as the sum of PG and MG.

Statistics

Data were analyzed using two-way ANOVA. For some experiments, the data were analyzed with a one-way ANOVA, when this was warranted, and when appropriate, a Fisher's LSD post hoc analysis was used. All data are reported as means ± SE.

RESULTS

Effect of palmitate on insulin-stimulated glucose transport

In control muscle insulin-stimulated glucose uptake was not altered during the 18h incubation period (P>0.05; Figure 1). In palmitate-treated muscle (0–18 h) insulin-stimulated glucose transport was markedly and progressively reduced (-33%, -66%, and -87%) over 6, 12 and 18 h of palmitate incubation (P<0.05; Figure 1) respectively.

When AICAR (2mM) was present during the last 6h of incubation with palmitate, the insulin stimulated glucose uptake was increased to levels observed in the control muscle (P<0.05; Figure 1), despite the presence of palmitate.

Basal glucose transport was not altered during the 18h incubation period either in the absence or presence of palmitate (data not shown). However, in AICAR-treated muscle, basal glucose transport was slightly altered during the 18-h incubation period (data not shown). However, these smaller changes in basal glucose transport cannot account for the much larger changes induced by AICAR in insulin-stimulated glucose transport.

### Effect of palmitate on fatty acid oxidation

The rate of palmitate oxidation was not altered in control muscles (P >0.05; Figure 2). In contrast, in the palmitate-treated muscles, palmitate oxidation was

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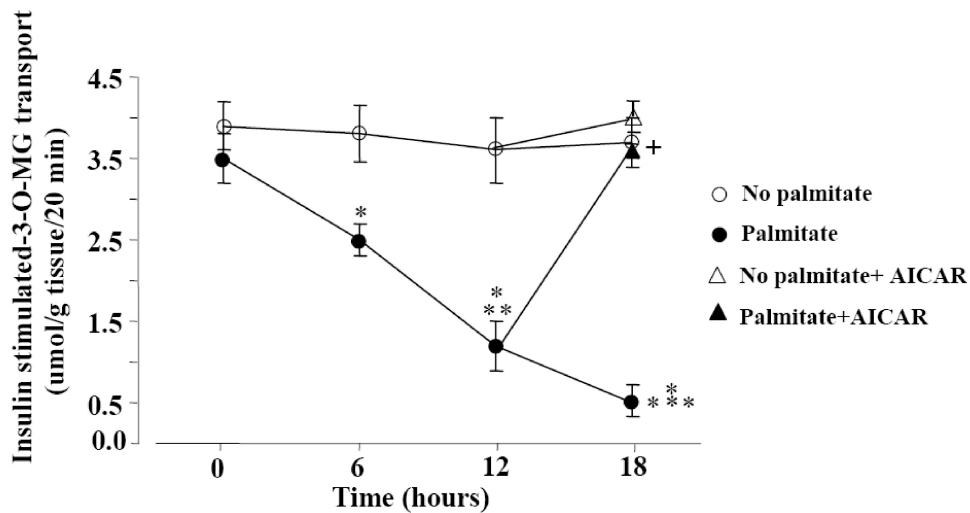
N=6-8 muscles per data point; *P<0.05 palmitate-treated muscles at t = 6, 12, and 18 h vs. 0h; **P< 0.05, palmitate-treated muscles at t = 12 vs. 6 h; ***P< 0.05, palmitate-treated muscles at t= 18 vs. 12h; + P< 0.05, palmitate-treated muscles + AICAR treatment at *t* = 18 h vs. palmitate-treated muscles at *t*=18h.

Figure 1 : Insulin-stimulated glucose transport in control and palmitate (2 mM) treated muscles (0–18 h), and in muscles that were treated with AICAR (2 mM) for 6 h (12–18 h) while high concentration of palmitate (2 mM) was maintained. Data are presented as means ± SE.
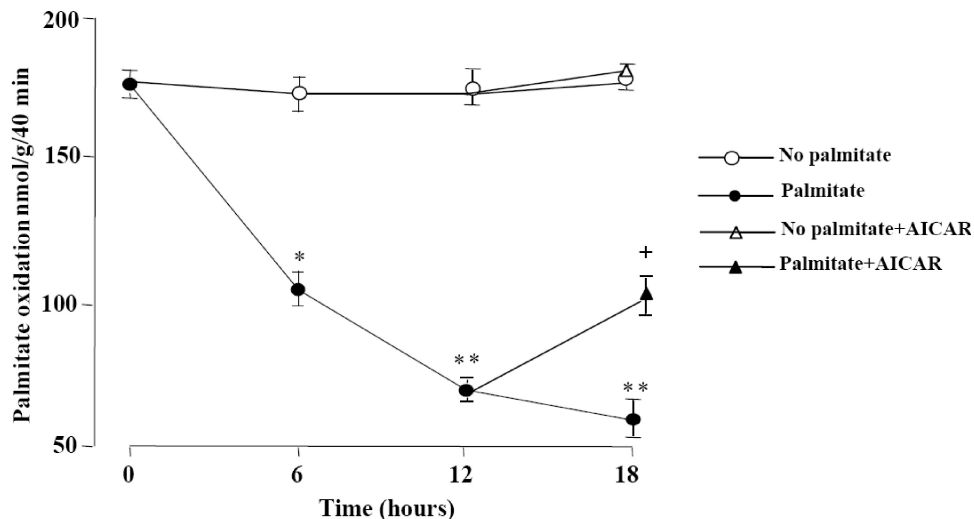
N=6-8 muscles per data point; *P<0.05 palmitate-treated muscles at t = 6, 12, and 18 h vs. 0h; **P< 0.05, palmitate-treated muscles at t = 12 vs. 6 h; + P< 0.05, palmitate-treated muscles + AICAR treatment at *t* = 18 h vs. palmitate-treated muscles at *t*=18h.

Figure 2 : Palmitate oxidation in control and palmitate (2mM) treated muscles (0-18 h), and in muscles that were treated with AICAR (2mM) for 6 h (12-18h) while high concentrations of palmitate (2 mM) were maintained. Data are presented as means ± SE.

markedly decreased by -40% at 6 h (P <0.05; Figure 2) and remained reduced (-50%) up to 12 h of incubation (P <0.05; Figure 2). No further reduction occurred during the 12-18h period (P>0.05; Figure 2).
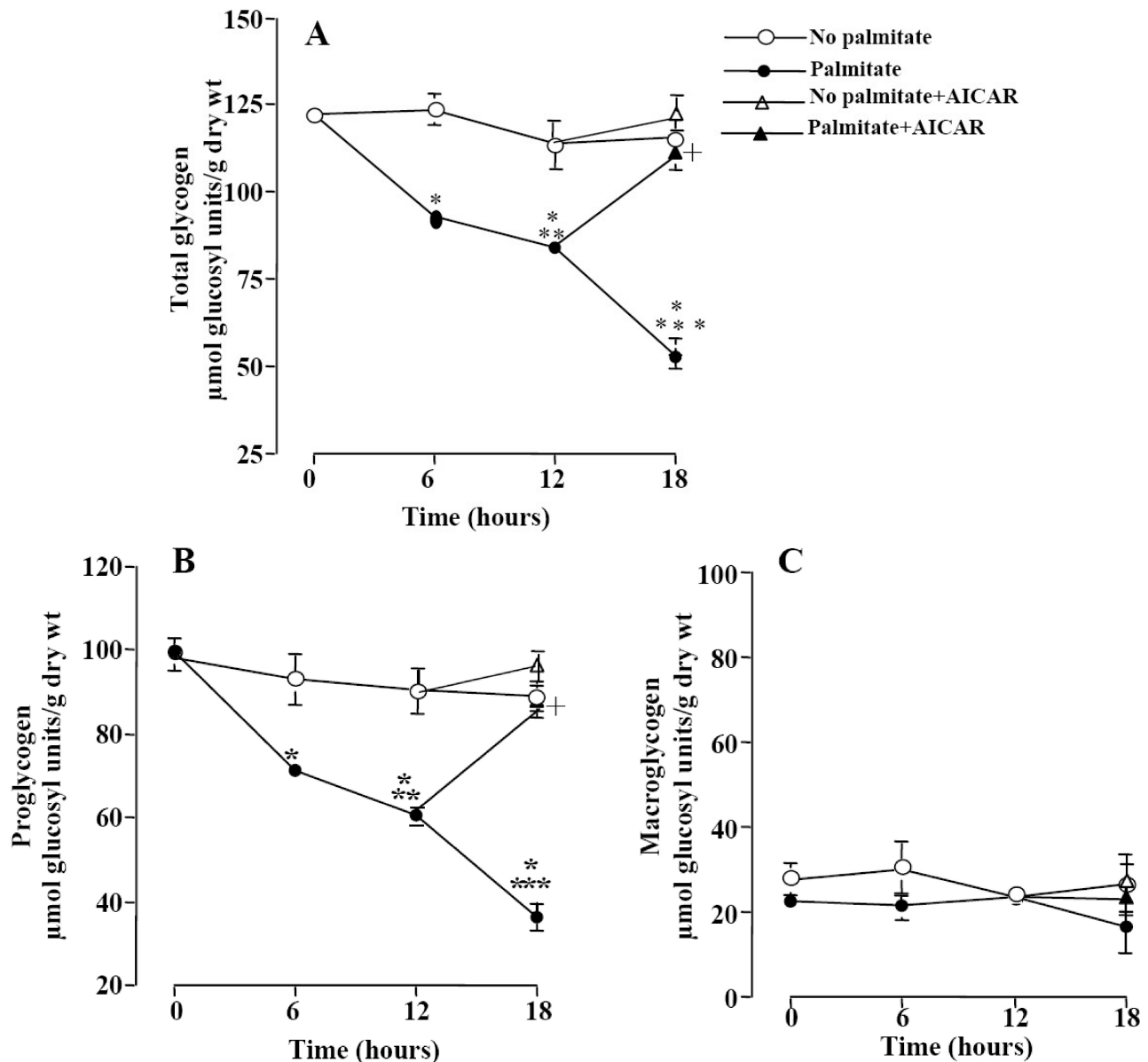
In the palmitate treated muscle, palmitate oxidation rates were markedly increased when muscles were treated for 6 h with AICAR (P<0.05; Figure 2) despite the continued presence of palmitate. However, these increased rates of palmitate oxidation remained lower

in the AICAR- treated muscles than in 18h control muscles (P< 0.05; Figure 2).

Basal palmitate oxidation was not altered with any of the experimental treatments (data not shown).

Characterization of muscle proglycogen or macroglycogen utilization

In control muscles, the total muscle glycogen concentration was not altered during incubation time (0-

N=6-8 muscles per data point; in some cases the error bars are less than the plot symbol; *P<0.05 palmitate-treated muscles at t = 6, 12, and 18 h vs. 0h; **P< 0.05, palmitate-treated muscles at t = 12 vs. 6 h; ***P< 0.05, palmitate-treated muscles at t= 18 vs. 12h; + P< 0.05, palmitate-treated muscles + AICAR treatment at *t* = 18 h vs. palmitate-treated muscles at *t*=18h.

Figure 3 : Total amount of glycogen (*A*), proglycogen (*B*), and magroglycogen (*C*) in control and palmitate (2 mM) treated muscles (0–18 h), and in muscles that were treated with AICAR (2 mM) for 6 h (12–18 h) while high concentrations of palmitate (2 mM) were maintained. Data are presented as means ± SE.

18h) (*P*>0.05, Figure 3A). This was attributed to the unaltered concentration of both the proglycogen (*P*>0.05, Figure 3B) and macroglycogen (*P*>0.05, Figure 3C) pools. In contrast, in the palmitate-treated muscles, total muscle glycogen was progressively reduced by -23%, -30%, and -57% (*P*<0.05, Figure 3A) at 6h, 12h and 18h respectively. This reduction was attributed to decrements in the proglycogen pool by -30% at 6h, -40% at 12h, and -62% at 18h

(*P*<0.05, Figure 3B). However, there was no change in the macroglycogen pool (*P*>0.05, Figure 3C).

The basal concentrations of proglycogen and macroglycogen were not altered with any of the experimental treatments (data not shown).

Effect of AICAR on muscle proglycogen and macroglycogen pools

In palmitate-treated muscles, AICAR treatment fully

rescued the total muscle glycogen to the levels observed in the untreated control muscles ($P<0.05$, Figure 1A). This was attributed to fully rescued proglycogen pool (P<0.05, Figure 1B) rather than macroglycogen pool (P>0.05, Figure 1C).

DISCUSSION

In the present study, we used our *in vitro* soleus muscle model that is metabolically viable for $\leq 18h$[22] to explore 1) the possible changes in proglycogen and macroglycogen pools under insulin resistance state, in which insulin-stimulated glucose uptake and fatty acid oxidation were experimentally reduced by palmitate treatment (2mM, 0-18h); and 2) the responses of these pools to rescue of insulin resistance by AICAR treatment.

Our study has provided novel findings on the effects of insulin resistance, or its amelioration by AICAR on proglycogen and macroglycogen pools. Specifically, we found that 1) induction of insulin resistance by prolonged incubation of soleus muscles with palmitate (2mM, 0-18h) reduced proglycogen pool but not macroglycogen pool; while 2) restoring insulin sensitivity, and fatty acid oxidation by AICAR treatment completely rescued proglycogen pool. Additionally, the data further suggest that 3) proglycogen and macroglycogen pools are metabolically regulated by different ways.

In agreement with our previous studies[17,18,21], this study showed that long incubation of solues muscle with palmitate (2mM, 0-18h) inhibited insulin stimulated glucose transport. This reduction was also accompanied by reduction in fatty acid oxidation. However, treatment with AICAR (6 h) completely ameliorated palmitate-induced insulin-resistance in the isolated soleus muscle, and the expected increase in fatty acid oxidation did occur.

The energy demands of the incubated soleus muscle are likely minimal, and hence, in absence of insulin resistance (control muscle), intramuscular glycogen was not diminished. Concomitantly, proglycogen and macroglycogen pools were very well maintained in these muscles. Therefore, in these muscle it is clear that glucose in the incubating media was sufficient to maintain an adequate energy supply, and substrate to support glycogen synthesis.

In the presence of insulin resistance (palmitate-treated muscle) there was a marked reduction in insulin-stimulated glucose uptake, this reduction in insulin action was closely associated with reduction in the oxidation of fatty acids. Concomitantly, there was a large reduction in muscle glycogen concentration. Presumably, this reduction in muscle glycogen is the result of a reduced capacity of muscle to oxidize palmitate to generate ATP, as well as a diminished capacity to take up glucose. The reduction in glycogen found in this study was mainly attributed to a reduction in the proglycogen pool, which is known to be more dynamic than the macroglycogen pool[5,6,8]. Therefore, it appears that in this insulin resistance model, energy was maintained by increasing proglycogen hydrolysis, as fatty acid oxidation and glucose uptake were impaired. Additionally, the recovery of insulin resistance, and fatty acid oxidation by AICAR treatment was accompanied by the concurrent rescue of muscle glycogen. This increase in muscle glycogen concentration was attributed to rescued of proglycogen pool.

Taken together, our findings suggest that hydrolysis of proglycogen pool represents an essential source of energy in an *ex vivo* model of insulin resistance that is characterized by reduction in insulin action and fatty acid oxidation. However, proglycogen hydrolysis may not be the only source of energy under such circumstances. For example, intramuscular lipids may contribute to maintain muscle energy. However, the possible contributing of intramuscular lipids to maintain muscle energy is beyond the scope of this study.

To the authors' knowledge, this is the first study to suggest that proglycogen and macroglycogen pools can be differently regulated in an *ex vivo* model of insulin resistance. The fact that proglycogen was broken down in favor of macroglycogen is not clear, and our data did not address this issue. However, this could be attributed to the fact that the proglycogen pool is more dynamic, and more sensitive than the macroglycogen pool[5-9]. In exercising muscle it is known that proglycogen provides much of the muscle's energy and this pool is also rapidly repleted when carbohydrates are provided after exercise[5,6,8].

In conclusion, this study suggests, for the first time, that proglycogen pool rather than macroglycogen pool is preferentially utilized in ex vivo model of insulin resistance, that is characterized by reduction in fatty acid oxidation and glucose utilization. This increase in the

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hydrolysis of proglycogen pool appeared to maintain stable muscular energy under such circumstances.

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