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**Metabolic Inflexibility Revisited: Muscle Substrate Oxidation Is Dissociated From Muscle Insulin Resistance**

Joongyu Daniel Song

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Metabolic Inflexibility Revisited:
Muscle Substrate Oxidation is Dissociated from
Muscle Insulin Resistance

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Medicine

By
Joongyu Daniel Song
2019
Abstract

Alterations in basal metabolic substrate preference were proposed as early derangements in the development of skeletal muscle insulin resistance. Here, we report that alterations in muscle substrate oxidation are not associated with muscle insulin resistance in rats or humans. In this study we used a novel stable isotope tracer ([U-13C]glucose) liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure the ratio of mitochondrial glucose oxidation by pyruvate dehydrogenase flux (V_{PDH}) relative to rates of citrate synthase flux (V_{CS}) in skeletal muscle. We found that high fat diet (HFD) fed insulin resistant rats did not have altered substrate oxidation in soleus muscle in the fasting state. Hyperinsulinemic-euglycemic clamps increased relative glucose oxidation in both normal and insulin resistant rats, although this increase was blunted in HFD fed insulin resistant rats, due to an impairment in insulin-stimulated muscle glucose transport. Additionally, we found that an acute infusion of lipid during a hyperinsulinemic-euglycemic clamp in normal rats significantly reduced mitochondrial V_{PDH}/V_{CS} flux in soleus muscle without any effects on insulin-stimulated peripheral glucose metabolism or muscle glucose transport. Using the same stable isotope/LC-MS/MS approach we then examined V_{PDH}/V_{CS} flux in insulin sensitive and insulin resistant humans and found similar relative rates of V_{PDH}/V_{CS} following an overnight fast and similar increases in V_{PDH}/V_{CS} fluxes during a hyperinsulinemic-euglycemic clamp. Taken together these results show that basal mitochondrial substrate preference in muscle is not altered in insulin resistant rats or insulin resistant humans and that acute modulation of substrate
oxidation in normal rats does not affect muscle insulin sensitivity refuting the metabolic inflexibility hypothesis which stipulates that alterations in substrate oxidation as an essential step in the development of muscle insulin resistance.
Acknowledgements

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I. **Insulin Resistance and Type 2 Diabetes**

Type 2 Diabetes (T2D) is emerging as a global epidemic, with the prevalence of the disease increasing at a rate far exceeding previous predictions (1,2), with close to 50 million people expected to live with T2D by the year 2030 (3). The age of onset of T2D has inched earlier, and the prevalence of the disease has increased among children and adolescents in recent years (4,5). These trends portend a distressing future: the United States alone spent more than $170 billion in 2007 for treatment of diabetes and its associated complications (6), and the disease is expected to exact a heavy cost from economies around the world.

Despite the widespread and increasing prevalence of T2D and the significant attention devoted to its study, the precise molecular mechanisms leading to the development of T2D remain relatively poorly characterized. T2D can be defined as the progressive development of insulin resistance accompanied by the failure of insulin secretion that culminates in the clinical manifestation of inappropriate fasting hyperglycemia. The exact sequence of these components – as well as the relative importance of each – continues to be debated, but growing evidence has pointed to the centrality of insulin resistance as the key event in the pathogenesis of T2D (7–9). Impaired glucose disposal in response to insulin is seen as one of the earliest defects in susceptible individuals (8–10). In fact, islet cells increase insulin secretion in the early stages of diabetes during a period of
compensated, subclinical insulin resistance (i.e. without overt hyperglycemia); when β-cells are no longer able to compensate – either from the severity of insulin resistance or due to a number of genetic factors that may predispose it to failure – the patient manifests clinically with diabetes (11,12). Thus, understanding insulin resistance and the precise molecular steps leading to its development is key to understanding T2D and to developing potential therapies.

As master regulator of metabolism, insulin accomplishes a variety to key tasks in disparate organ systems, which include 1) the suppression of lipolysis (or the breakdown and release of fatty acids) in adipose tissue, 2) the shift from gluconeogenesis to glycogen storage and lipogenesis in the liver, and 3) the stimulation of glucose uptake and glycogen storage in skeletal muscle (7). These varied effects ultimately converge to aid the body in the fasting to fed transition; from the reliance on endogenous glucose production (performed by the liver and crucial for obligate glucose utilizers like the brain) and fat/ketone oxidation (which occurs in peripheral tissues like skeletal muscle) during times of nutrient deprivation, to the storage and usage of exogenous glucose in times of nutrient abundance (7,13). Insulin resistance, put simply, is the inability of insulin to produce these effects in its target cells.

Skeletal muscle is a particularly important site of insulin action, as well as a crucial player in the development of insulin resistance and T2D. For one, skeletal muscle has a disproportionate influence on whole-body glucose homeostasis, as it accounts for a major fraction of insulin-stimulated extrahepatic glucose uptake (10). Furthermore, abnormalities in skeletal muscle – such as disordered nutrient
storage, intramyocellular ectopic lipid accumulation, and associated impairments in insulin-stimulated glucose uptake – have been observed in predisposed individuals decades before the onset of frank T2D (8,14–16), suggesting that derangements in skeletal muscle are some of the earliest to occur in the pathogenesis of T2D, perhaps even earlier than dysfunctions in hepatic insulin response. Third, exercise has been shown to stimulate glucose uptake in insulin resistant muscle in an insulin-independent manner (17–19), emphasizing skeletal muscle’s current and potential role (through both behavioral and pharmaceutical approaches) in the treatment of T2D.

Despite intense study, the precise location of muscle insulin resistance – that is, the molecular defect that prevents insulin from eliciting its normal physiologic response in muscle – remains an unsolved mystery. Many of insulin’s physiologic intracellular actions have been characterized: insulin’s binding to its receptor sets off a cascade of events in the myocyte including heterodimerization and autophosphorylation of the insulin receptor (a member of the tyrosine kinase receptor family) and recruitment of key downstream mediators such as insulin receptor substrate-1 (IRS-1), phosphoinositide-3 kinase (PI3K), and AKT. These events ultimately serve to increase the translocation of the glucose transporter GLUT4 to the membrane, increase glucose influx into the cell, increase the rate of glycolysis, and increase glycogen synthesis (20–24). Many of these steps (as well as other steps in insulin-independent pathways) have been implicated in the development of muscle insulin resistance.
II. Muscle Substrate Oxidation and the Metabolic Inflexibility Hypothesis

In this setting, alterations in mitochondrial substrate preference and oxidation have been hypothesized as essential derangements in skeletal muscle insulin resistance. Mitochondria are the site of oxidative disposal of acetyl-CoA, the end product of both glycolysis and β-oxidation of fatty acids. In a seminal study published more than 50 years ago, Randle et al. proposed that skeletal muscle glucose and fat oxidation existed in competition within the myocyte and that disordered oxidation of fat could in turn effect intracellular handling of glucose. Based on a series of in vitro experiments in the rat heart, Randle observed that increasing fat oxidation in the myocyte could directly inhibit glycolysis by increasing the levels of key metabolites that could allosterically inhibit enzymes in the glycolytic pathway, ultimately leading to the inhibition of glucose uptake into the cell. From these observations, Randle proposed that this intracellular glucose-fatty acid cycle (nicknamed the “Randle cycle”) explains how 1) in normal individuals, the physiologic increase in the availability of fatty acids in the fasting state leads to an increase in fat oxidation and concomitant decrease in glucose oxidation in myocytes and 2) in insulin resistant individuals, pathologically elevated fat oxidation could inhibit glucose oxidation and, in turn, glucose uptake in myocytes (25) (Graphic 1). The hypothesis notably did not require the implication of defects in intracellular insulin signaling; increased fat oxidation (either directly through altered mitochondrial preference, or indirectly through increased delivery of fatty
acids to the myocyte) could reduce muscle glucose uptake regardless of changes in insulin signaling. While Randle’s observations and predictions failed to be confirmed in subsequent in vivo experiments, the ‘Randle cycle’ represented in its time a novel and attractive explanation that connected mitochondrial substrate handling and whole-body glucose homeostasis.

More recently and in a similar vein, the concept of ‘metabolic inflexibility’ has been proposed to cause muscle insulin resistance. The metabolic inflexibility hypothesis, proposed by David Kelley, also placed disordered mitochondrial substrate handling as the locus of insulin resistance, rather than defects in the insulin signaling cascade. In his in vivo experiments in humans, Kelley used leg-specific indirect calorimetry techniques¹ to measure muscle substrate oxidation and compare them between normal and insulin resistant subjects. Kelley found that while the muscles of normal individuals relied predominantly on fat oxidation in the fasting state and on glucose oxidation in the insulin-stimulated state, insulin resistant individuals 1) had paradoxically increased preference for glucose oxidation in the fasting state and 2) could not modulate their muscle substrate preference in response to insulin (26,27). Stated another way, insulin resistant muscle seemed to be ‘locked in’ to a particular ratio of glucose to fat oxidation and could not adjust its oxidative fuel based on physiological

¹ Indirect calorimetry refers to the measurement of the arteriovenous differential in oxygen and carbon dioxide content (in this case, across the leg) to approximate relative substrate oxidation in myocytes. The technique centers on knowing the predicted stoichiometries of oxygen consumption and carbon dioxide production for the oxidation of specific substrates (glucose, fat, ketone, amino acid). In the case of humans in normal physiologic conditions, glucose and fatty acids were assumed to be the major oxidative substrates in muscle.
circumstance, fasting or fed. Kelley went on to conclude from these observations that insulin resistance was a manifestation of underlying cellular ‘metabolic inflexibility,’ or the inability to alter substrate oxidation in varying physiologic states: metabolically inflexible myocytes operated at a saturated rate of glucose oxidation that created an intracellular backlog unfavorable to further glucose uptake, even in the setting of insulin (28). He further hypothesized that defects in the mitochondrial enzymatic machinery that regulates both basal substrate metabolism and transitions between glucose and fat oxidation were mechanistically responsible for the onset of diabetes.

The concept of metabolic inflexibility has gained considerable traction in the field of metabolism, and recent studies have proposed aberrations in pyruvate dehydrogenase (PDH) (29), pyruvate dehydrogenase kinase (PDK) isoforms (30), carnitine-palmitoyl transferase-1 (31), and carnitine acetyltransferase (CrAT) (32) as possible mediators of altered basal substrate handling in insulin resistant muscle and, therefore, as potential therapeutic targets in T2D.

(Continued on page 13)
A schematic of the proposed Randle cycle, which shows that increased fat oxidation results in an increase in acetyl-CoA and citrate, which in turn inhibits key enzymes in the glycolysis pathway.

**Graphic 1.** A schematic of the proposed Randle cycle, which shows that increased fat oxidation results in an increase in acetyl-CoA and citrate, which in turn inhibits key enzymes in the glycolysis pathway.
III. Problems with the Metabolic Inflexibility Hypothesis and Alternatives

Despite its widespread acceptance, whether metabolic inflexibility could explain tissue-specific insulin resistance in vivo remains to be confirmed. In fact, the indirect calorimetric technique employed in Kelley’s original studies have significant methodological shortcomings that preclude making definitive conclusions about tissue-specific substrate oxidation and changes therein. For one, measurements of respiratory quotient using arterio-venous (A-V) respiratory quotient (RQ) measurements across the leg were not truly muscle-specific but reflect glucose and fat oxidation in additional tissues such as fat and bone. Further, indirect calorimetry captures substrate disappearance from blood rather than true cellular oxidation and does not account for interconversions between metabolic substrates that occur in vivo and thus, is an imperfect measurement for true substrate oxidation in the cell (33). To date, no conclusive evidence exists that insulin resistant muscles have altered substrate preference in the fasting state and that the blunting of insulin’s ability to increase muscle glucose oxidation is a result of oxidative inflexibility rather than a primary defect in insulin-mediated glucose transport.

In fact, evidence suggests that the defect in muscle insulin response in T2D occurs at the level of insulin response, with impairments in the ability of insulin to increase glucose transport across the membrane. A series of in vivo human studies utilizing non-invasive magnetic resonance spectroscopy demonstrated that
muscles of individuals had impaired glycogen synthesis not as a result of a ‘backlog’ and allosteric inhibition from disordered downstream oxidation, but from a deficiency of substrate secondary to impaired insulin-stimulated GLUT4 translocation and defective glucose transport across the sarcolemma (34–37). Furthermore, significant accumulation of ectopic intramyocellular lipid has been observed in the early stages of insulin resistance in individuals susceptible to T2D; subsequent experiments showed that this ectopic lipid deposit increases intracellular concentrations of bioactive lipid species such as diacylglycerols (DAG), which in turn activates novel protein kinase C (PKC) isoforms that interfere significantly with the intracellular insulin signaling transduction, particularly at the level of insulin receptor substrate 1 (IRS1) (38–42). Taken together, these studies suggest that, contrary to the metabolic inflexibility hypothesis, the primary molecular insult in muscle insulin resistance is a proximal impairment in insulin response and glucose transport secondary to excessive caloric intake and ectopic lipid accumulation, rather than a backlog mechanism from a distal inflexibility in mitochondrial substrate handling.

IV. Aims and Hypothesis

To directly assess the metabolic inflexibility hypothesis and avoid the limitations of the A-V indirect calorimetry methodology we applied a novel combined stable isotope tracer/LC-MS/MS approach to capture true muscle-specific substrate oxidation and examine whether ‘metabolic inflexibility’ is operating in an in vivo model of insulin resistance. By infusing [¹³C₆] glucose and analyzing ¹³C
enrichments of alanine and glutamate isotopologues in muscles, we measured
mitochondrial pyruvate dehydrogenase flux ($V_{PDH}$) as a proportion of mitochondrial
citrate synthase flux ($V_{CS}$), a highly sensitive and tissue-specific index for the
relative contribution of glucose oxidation to total mitochondrial oxidation
($V_{PDH}/V_{CS}$). Using this method, we studied how induction of insulin resistance in
rats by high fat feeding affects substrate preference in the fasted state as well as
during a hyperinsulinemic-euglycemic clamp, which simulates the fed state. In
addition, we tested whether acute infusion of lipid during a hyperinsulinemic-
euglycemic clamp, thus putting glucose and fatty acids in direct competition, in
normal rats could acutely modulate substrate oxidation in vivo and whether these
modulations in muscle oxidative substrate preference correlated with changes in
whole body insulin sensitivity.

We hypothesized that:

1. The major oxidative substrate during fasting is fat, not glucose.
2. Oxidative substrate preference in the fasting state in skeletal muscle is not
different between normal and insulin resistant subjects.
3. Insulin increases glucose transport, glycolytic flux, and glucose oxidation in
   normal subjects. In insulin resistant subjects, the increase in glucose
   oxidation is blunted.
4. Failure to increase glucose oxidation in response to insulin in insulin-
   resistant subjects is due to impaired insulin-mediated glucose transport, not
due to metabolic inflexibility or due to defects in cellular oxidative
   machinery.
Methods

I. Experimental Design

The study design consisted of two rat populations, one fed regular chow and the other fed four weeks of a high fat diet (HFD) that induces insulin resistance. Surgical placement of arterial and venous catheters enabled infusions of various tracers and bioactive agents in live, awake, and non-stressed rats, as well as sampling of venous blood for the measurement of real-time concentrations of metabolites. This design allowed us to study of dynamic changes in whole-body in vivo metabolism rather than relying on in vitro or partial systems. Insulin sensitivity/resistance was measured using hyperinsulinemic-euglycemic clamps, in which a standardized rate of insulin and variable rates of glucose are infused together in the rats to maintain euglycemia (~100 mg/dL) and the rate of glucose required provides a quantitative index of the animal’s sensitivity to insulin. To study muscle substrate oxidation, normal and HFD-fed rats were given a two-hour trace infusion of glucose uniformly labeled with $^{13}$C (i.e. all six of its carbons are $^{13}$C); the infusion of trace amounts ensured that the exogenous infusion of glucose did not reflect a substantive increase in total substrate seen by the tissues of interest, while the two-hour infusion time enabled the concentrations of tracer and its downstream metabolites to reach steady state in blood and tissues. The infusions of the glucose tracer were performed both after an overnight fast (which we designated as the animal’s basal state), and concomitantly during a hyperinsulinemic-euglycemic clamp (which simulated the hyperinsulinemic conditions of the fed state). At the conclusion of the infusions, the rats were
sacrificed and tissues of interest were quickly harvested and freeze-clamped in liquid nitrogen to minimize post-mortem/ischemic changes.

In order to measure relative rates of glucose and fat oxidation in skeletal muscle, tissue samples were analyzed for enrichments of in downstream intracellular metabolites of the labeled glucose. Enrichment is defined as the proportion of a labeled isotope to its native, non-labeled form. In the case of our study, the infused glucose tracer mixes in blood with endogenous unlabeled glucose, and the mixed pool of endogenous and isotopic glucose is taken up by the myocyte. In the myocyte, the mixed glucose pool undergoes glycolysis and the resulting acetyl-coA participates in the tricarboxylic acid cycle in mitochondria. The downstream metabolites produced as a result of these intracellular glycolytic and oxidative processes exist in mixed pools of native molecules and isotopes (i.e. endogenous glucose will produce unlabeled acetyl-coA with glycolysis, while the tracer will produce acetyl-coA with both of its carbons labeled with $^{13}$C), and the particular enrichments of each downstream metabolite depends on the starting enrichment of the upstream substrate, as well as any subsequent dilutions from endogenous metabolites contributed by other intracellular pathways. As an example, the major processes that contribute to the intracellular acetyl-coA pool include glycolysis, beta oxidation of lipids, and breakdown of ketones. While with an infusion of glucose tracer glycolysis will increase the pool of labeled acetyl-coA, any acetyl coA produced from beta oxidation or ketone oxidation will result in the dilution of the enrichment of the intracellular acetyl-coA pool (Graphic 3). Insofar as we can measure the enrichments of various metabolites and have a working
model of the major cellular reactions contributing to the production, modification, and degradation of these metabolites, we can relate the measured enrichments to the rate of the reactions affecting them.

In a previous study, we showed that the using the ratio of measured enrichments in select isotopes of alanine and glutamate in the tissues samples of rats infused with labeled glucose tracer could be used to approximate the ratio of *in vivo* glycolytic flux to the total oxidative flux ($V_{PDH}/V_{CS}$), or the relative contribution of glucose oxidation to total oxidation in the myocyte (43). Theoretically, calculating $V_{PDH}/V_{CS}$ requires measurement of enrichments of intracellular pyruvate and acetyl-coA, as the enzyme pyruvate dehydrogenase converts pyruvate into acetyl-coA and is the final link between glycolysis and the TCA cycle. In reality, intracellular pyruvate is difficult to measure consistently, while intracellular acetyl-CoA is prone to rapid postmortem ischemic degradation. Therefore, in conditions of steady state with infusion of our tracer, we assumed that intracellular pyruvate enrichment equilibrates with the enrichment of alanine (a related three-carbon molecule produced by the transfer of an amino group from pyruvate), while the acetyl-coA enrichment equilibrates with α-ketoglutarate, which in turn equilibrates with the enrichment of the glutamate pool (in particular, glutamate enriched at the 4,5 carbon positions, the expected enrichment positions of any $^{13}$C contributed by the acetyl-coA derived from the infused tracer). Alanine and glutamate form stable intracellular pools in steady state that can be measured consistently and is relatively resistant to rapid postmortem changes (Graphic 4). However, in order to definitively show that alanine and glutamate enrichments
would serve as appropriate surrogates for pyruvate and alanine, respectively, we performed a series of validation experiments comparing the
In tissue samples, the ratio of measured glutamate (m+2) enrichment to alanine (m+3) enrichment reflects the ratio of glucose oxidation to total mitochondrial oxidation ($V_{PDH}/V_{CS}$). A $V_{PDH}/V_{CS}$ ratio of 0 results from 100% fat oxidation relative to total oxidation, while a ratio of 1 suggests sole contribution of glucose oxidation to total oxidation.

**Graphic 3.** A schematic demonstrating the conceptual basis for our tracer approach for determining tissue-specific substrate oxidation. The infused [$^{13}$C]$\_6$ glucose is taken up by the muscle cell, and the labeled carbons assume predictable positions in downstream metabolites. The pool of labeled acetyl-CoA from the glucose tracer is diluted by contributions from beta oxidation of fatty acids, such that measurement of proportional labeling (i.e. enrichment) of particular metabolites before and after this dilution can be used to determine relative glucose and fat oxidation rates at the cellular level. The schematic also shows our assumption that pyruvate and $\alpha$-ketoglutarate enrichments equilibrate with alanine and glutamate enrichments, respectively.

**Graphic 4.** In tissue samples, the ratio of measured glutamate (m+2) enrichment to alanine (m+3) enrichment reflects the ratio of glucose oxidation to total mitochondrial oxidation ($V_{PDH}/V_{CS}$). A $V_{PDH}/V_{CS}$ ratio of 0 results from 100% fat oxidation relative to total oxidation, while a ratio of 1 suggests sole contribution of glucose oxidation to total oxidation.
enrichments of these pools in a variety of physiologic conditions, including fasting and hyperinsulinemic states.

Using the above approach, we measured tissue-specific substrate oxidation (i.e. $V_{PDH}/V_{CS}$) in skeletal muscle for both fast-twitch and slow-twitch skeletal muscle. We studied how substrate oxidation changes compares between normal and HFD-fed insulin resistant rats, during both fasting and fed-simulated (i.e. hyperinsulinemic) conditions. To further test the validity of the metabolic inflexibility hypothesis and to see whether there was metabolic ‘overload’ occurring in the mitochondria in insulin resistant muscle, we measured levels of key oxidative enzymes (pyruvate dehydrogenase, pyruvate dehydrogenase kinase, carnitine palmitoyl transferase, and carnitine o-acetyl transferase) in tissues of both normal and HFD-fed rats. To study the changes in insulin signaling with insulin resistance, we measured levels and activities (as indicated by the proportion of phosphorylated to total levels) of key enzymes in the insulin signaling cascade, as well as concentrations of lipid species previously shown to interfere with insulin signaling.

To study the effect of acute modulation of muscle substrate oxidation and its relation to insulin resistance, we studied another cohort of normal rats and compared them under two conditions, one during a hyperinsulinemic-euglycemic clamp, the other during a co-infusion of non-esterified fatty acid during a hyperinsulinemic-euglycemic clamp. Insulin is a powerful suppressor of endogenous lipolysis; thus, the control group saw almost no fatty acid, while co-infusion of fatty acid during the clamp raised the level of plasma fatty acid to fasting
levels and provide and increase fatty acid oxidation in skeletal muscle. As previously with our comparison of normal and HFD-fed rats, we performed infusions of uniformly-labeled glucose tracer, measured $V_{\text{PDH}}/V_{\text{CS}}$ in skeletal muscle samples, measured levels of key oxidative enzymes as well as mitochondrial metabolites, and measured the phosphorylation of key enzymes in the insulin signaling cascade.

Further information about the particular materials and specific protocols used to undertake components of the above-mentioned overarching experimental design are listed in detail below. Included at the conclusion of the methods section is a statement about which components of the experiment were performed by the author and which were performed by collaborating scientists.

II. Experimental Model And Subject Details

All animal studies were approved by the Yale University Institutional Animal Care and Use Committee and were performed in accordance with all regulatory standards. 250 g male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and were group housed (3 per cage) while they were fed either a high fat diet (Dyets #112245, Bethlehem, PA; 59% calories from fat, 26% from carbohydrate, 15% from protein) or a chow diet (Harlan Teklad #2018, Madison, WI; 18% calories from fat, 58% from carbohydrate, 24% from protein), ad lib for 3 weeks. All rats then underwent surgery under general isoflurane anesthesia for placement of polyethylene catheters in the common carotid artery (PE50 tubing, Instech Solomon, Plymouth Meeting, PA) and the
jugular vein (PE90 tubing, Instech), after which they were singly housed. The rats were fed their respective diets ad lib for 1 more week, after which they were studied and sacrificed. All in vivo studies were performed following an overnight (16 hr) fast. At the conclusion of each study, rats were euthanized by IV pentobarbital.

III. Method Details

Animal Studies

In all studies, tracers were infused through a catheter placed ∼1 week prior in the carotid artery, and blood was obtained from a catheter in the jugular vein. Unless otherwise specified, all blood was drawn from the jugular vein. All studies began 1 hr after catheters were connected, reducing any impact of stress from handling on the physiology assessed.

Flux Analysis

To measure $V_{PDH}/V_{CS}$ flux in the basal fasted state, rats were infused with $[1,2,3,4,5,6^{-13}C_6]$glucose (prime 3 mg/[kg-min] for 5 min, continuous infusion rate 1 mg/[kg-min]) for a total of 120 min, after which blood (600 µl whole blood) was obtained from the venous catheter and immediately centrifuged and the plasma obtained, and rats were sacrificed and their soleus and quadriceps muscles snap-frozen in situ using metal tongs pre-chilled in liquid N₂. The $V_{PDH}/V_{CS}$ flux was measured as the ratio of $[4,5^{-13}C_2]$glutamate / $[^{13}C_3]$alanine in soleus and quadriceps after a 2 hr infusion of $[1,2,3,4,5,6^{-13}C_6]$glucose (16.7 µmol/[kg-min] prime for 5 min, 5.6 µmol/[kg-min] continuous infusion) based on the assumptions
we have described previously (43–45). Alanine enrichment was measured by GC/MS: samples were deprotonized with 5 volumes of methanol and derivatized with 3 volumes of b-butanol 4N HCl, after which the samples were heated to 65°C for 60 min, evaporated under N₂ gas, and resuspended in 100 μL of trifluoroacetic acid:methylene chloride (1:7). GC-MS was then used to determine the m+4 alanine enrichment (retention time ~4.1 min, m/z 242 [m0], 243 [m+1], 244 [m+2], 245 [m+3], 246 [m+4]). Glutamate enrichment was measured by LC-MS/MS: the samples were homogenized in 500 μL ice-cold methanol using a TissueLyser and filtered through a Nanosep filter. LC-MS/MS (AbSCIEX 6500 QTRAP with a Shimadzu ultrafast liquid chromatography system, negative ion mode) was used to monitor the relevant ion pairs: [m0] C4-5 glutamate, 146/41, [m+1] C4-5 glutamate, 147/47, and [m+2] C4-5 glutamate, 148/48.

To measure muscle glucose uptake, a bolus of [6,6-¹⁴C]2-deoxyglucose was administered in all rats. The harvested soleus and quadriiceps muscles were processed to determine glucose uptake in both tissues by comparing the plasma [¹⁴C] specific activity decay curve to tissue [¹⁴C] specific activity, both measured using a scintillation counter (46).

**Hyperinsulinemic Glucose Clamps**

Rats were given a 40 mU/kg bolus of Regular insulin followed by a 2 hr insulin infusion at a rate of 4.0 mU/(kg-min), while euglycemia was maintained with variable infusion of [1,2,3,4,5,6-¹³C₆]glucose. Blood samples were drawn from the venous catheter at 0, 15, 30, 45, 60, 70, 80, 90, 100, 120 min of the clamp, with
the samples from the 120 min time point used to measure clamp glucose turnover. Plasma insulin was measured by radioimmunoassay by the Yale Diabetes Research Center at the 0 and 120 min time points of the clamp. Glucose turnover in the clamp was calculated as above: $\text{Turnover} = \left( \frac{\text{Tracer enrichment}}{\text{Plasma enrichment}} - 1 \right) \times \text{Infusion rate}$, and the rate of whole-body glucose disposal was calculated as the sum of the glucose infusion rate plus the glucose turnover rate measured in the clamp.

For combined lipid infusion and hyperinsulinemic-euglycemic clamps, rats were infused with Intralipid 20% emulsion (Baxter Inc.) at the rate 40 µL/[kg-min] in addition to variable infusions of [1,2,3,4,5,6-13C6]glucose tracer for 2 hours. Plasma samples from 0 and 120 min time points for 2 hr lipid infusions were used to measure plasma non-esterified fatty acid levels with the method described below.

**Biochemical Analysis**

Plasma glucose concentrations were measured enzymatically using the YSI Glucose Analyzer (Yellow Springs, OH). Plasma NEFA concentrations were measured spectrophotometrically using a Wako reagent (Wako Diagnostics, Mountain View, CA).

**Tissue Analysis**

Muscle DAG and ceramide concentrations (40), acetyl- and malonyl-CoA (47,48) were measured as we have described. Muscle PKCθ membrane/cytosol ratio (49),
insulin receptor phosphorylation relative to total insulin receptor, and Akt phosphorylation relative to total Akt were measured by Western blot using antibodies from Cell Signaling. PDH phosphorylation relative to total PDH was measured by Western blot using antibodies from Calbiochem (pPDH s300, s232, s293) and Abcam (PDH). Glucose-6-phosphate concentrations were measured using an enzymatic assay from Sigma-Aldrich. Pyruvate concentrations were measured using GC/MS after derivatization with o-phenylene-diamine (Sigma-Aldrich) dissolved in 4M HCl, extraction with ethyl acetate, and further derivatization of the dried organic layer with a 1:1 BSTFA (Sigma-Aldrich) and pyridine (Sigma-Aldrich) mixture. Concentrations of TCA cycle intermediates (citrate, malate, succinate) were measured as reported previously (45). IRS-1-associated PI3-kinase activity was measured as reported previously (40). CPT1b was measured using antibodies from Santa Cruz. CrAT was measured using antibodies from ProteinTech. PDH, CPT1b, and CrAT levels were normalized to GAPDH levels, which was measured with antibodies from Cell Signaling. Muscle concentrations of G-6-P, pyruvate, acetyl-CoA, malonyl-CoA, and TCA cycle intermediates were measured in tissues freeze-clamped in situ to minimize changes due to post-mortem ischemia.

**IV. Quantification And Statistical Analysis**

Comparisons were performed using the 2-tailed Student's t-test, unpaired unless otherwise specified in the figure legends, with significance defined as a p-value <0.05. GraphPad Prism 7.0 (San Diego, CA) was used for all statistical analysis.
In most cases, n=8-10 rats per group, unless otherwise indicated in the figure legends. Data are presented as the mean±S.E.M.

V. Contributions

All animal experiments were designed/conducted and all data were analyzed independently by the author during the 2017-2018 academic year, with financial support from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), under the guidance of Dr. Gerald I. Shulman, Dr. Gary Cline, and Dr. Kitt F. Petersen. Preliminary experiments demonstrating the feasibility of using uniformly-labeled glucose tracer for the study of tissue-specific \( V_{\text{PDH}}/V_{\text{CS}} \) were performed by Tiago Alves, a former graduate student in the Shulman lab. The author learned how to perform clamps and tracer infusion experiments under the tutelage of Rachel Perry, a former postdoctoral fellow in the Shuman lab. Rachel Perry and graduate students Alexander Munk and Ye Zhang provided technical assistance during clamp and infusion experiments.

The following contributions were from staff scientists in the Shulman lab. Gina Butrico and Xian-Man Zhang provided assistance with the use of mass spectrometry (both liquid and gas chromatography) for the measurement of intracellular metabolite concentrations and their enrichments. Mario Kahn provided assistance with measurement of intramyocellular lipid species. Dongyan Zhang
instructed the author on western blot technique and assisted with the PI3K assay. Jianying Dong performed all of the surgeries on rats.
Results

In comparison to normal chow fed rats, rats fed 4 weeks of a high fat diet (HFD) exhibited increased body weight (Fig. S1A) and became insulin resistant, as manifested by higher plasma insulin levels required to maintain the same level of plasma glucose in the fasting state (Fig. S1B, D) as well as by reduced glucose infusion rate required to maintain euglycemia during a hyperinsulinemic-euglycemic clamp (Fig. S1G). Furthermore, insulin-stimulated muscle glucose uptake (Rd) and muscle glucose transport, as measured by radioactive 2-deoxyglucose uptake, were significantly impaired during the clamp (Fig. 1A-B), confirming the presence of skeletal muscle insulin resistance in these rats.

To test whether the metabolic inflexibility hypothesis could explain the development of muscle insulin resistance in these HFD-fed rats, we sought to study whether the HFD-fed rats had altered oxidative substrate preference in skeletal muscle in the basal state. We used $[^{13}\text{C}_6]$ glucose as a tracer during the clamp and used tissue-specific enrichments of alanine and glutamate to measure in vivo $V_{\text{PDH}}/V_{\text{CS}}$ in soleus and quadriceps muscles (43). As mentioned in the methods section, although calculation of true $V_{\text{PDH}}/V_{\text{CS}}$ requires the comparison of intracellular pyruvate and acetyl CoA enriched by the $[^{13}\text{C}_6]$ glucose tracer, these metabolites undergo rapid ex vivo degradation and are difficult to measure. Under conditions of steady state, intracellular pyruvate [m+3] was assumed to equilibrate with alanine [m+3], and acetyl CoA [m+2] was assumed to equilibrate with C4C5 glutamate [m+2]. Both alanine and glutamate are more stable ex vivo relative to their respective counterparts and were more amenable to reliable and consistent
measurement of their intracellular enrichments. A rigorous comparison study of the enrichments of these metabolites (performed in rat tissues freeze-clamped in situ to minimize ischemic degradation) showed significant and near 1:1 correlations between alanine and pyruvate enrichments as well as between C4C5 glutamate and acetyl CoA enrichments under a variety of experimental conditions (Fig. S3A-B), validating our equilibration assumptions and indicating that alanine and C4C5 glutamate enrichments could be reliably used to approximate in vivo $V_{PDH}/V_{CS}$.

Using this approach, we found that, contrary to what Kelley et al. had observed in humans using, A-V indirect calorimetry, skeletal muscles of normal rats relied predominantly (>90%) on fat oxidation in the fasting state (Fig. 1C-F) (27,50). Furthermore, in contrast to what the metabolic flexibility hypothesis would predict, we found that this overwhelming preference for fat oxidation in both soleus and quad muscle during the fasting state did not change in insulin resistant HFD-rats (Fig. 1C-F). In response to insulin during a clamp, normal rats upregulated relative glucose oxidation in soleus muscle to ~50% and in quad muscles, which contain a lower proportion of slow twitch (oxidative) to fast twitch (glycolytic) fibers, to ~25% (Fig. 1C-F). In HFD-fed rats, however, this increase in glucose oxidation in response to insulin was blunted in both skeletal muscles (Fig. 1C-F). These results indicated that insulin resistance in the HFD-fed rats was characterized not by alterations in basal substrate use but by a defect in their ability to increase glucose oxidation in the presence of insulin.

This impairment in the ability of HFD-fed rats to increase glucose oxidation with insulin could have resulted either from a defect in the mitochondrial oxidative
capacity or from limited substrate availability secondary to a defect in insulin-mediated glucose transport. To see which was operating in our rat model of insulin resistance, we measured basal levels of key oxidative enzymes and found that there were no differences in the expression of PDH, CPT1b (the isoform of CPT predominant in skeletal muscle), and CrAT, a regulator of the mitochondrial acetyl-CoA pool that was suggested as a candidate for altered substrate handling in the metabolic inflexibility hypothesis of insulin resistance (Fig. 2C). Furthermore, we found no difference in the phosphorylation of PDH in both fasting and clamped states between the two groups, suggesting that HFD-fed rats did not have impaired activity of PDH and capacity to oxidize glucose (Fig. 2A). Finally, muscle concentrations of tricarboxylic acid cycle metabolites were not altered with HFD (Fig. 2B), making it unlikely that the mitochondria of insulin resistant rats had ‘metabolic overload’ that might impede smooth transitions between fat and glucose oxidation. All in all, we did not find evidence that the mitochondria of HFD-fed rats acquired defects in mitochondrial oxidative capacity that could explain the impairments in insulin-stimulated glucose oxidation we observed in skeletal muscle. To test whether impairments in glucose transport could provide an alternative explanation, we measured previously reported mediators of ectopic lipid-induced blockage of insulin signaling and found that HFD-fed rats displayed increased baseline translocation of protein kinase Cθ (PKCθ) and impaired phosphorylation of AKT in response to insulin (Fig. 2D,F). Furthermore, HFD-fed rats showed significantly reduced insulin receptor substrate-1 (IRS-1)-associated phosphoinositide 3-kinase (PI3-K) activity, a proximate and sensitive indicator of
insulin-stimulated glucose transport (Fig. 2E). Altogether, these results support the hypothesis that the major defect in the early stages of the pathogenesis of muscle insulin resistance is ectopic lipid-induced blockage in insulin-mediated glucose transport due to proximal defects in insulin signaling, rather than acquired 'metabolic inflexibility.'

Given that insulin resistant rats fed HFD failed to display alterations in basal substrate use, we wanted to see if acute modulation of intramyocellular substrate oxidation in normal rats could influence muscle insulin response. Hyperinsulinemic-euglycemic clamps suppressed fasting white adipocyte tissue (WAT) lipolysis as exhibited by reductions in non-esterified fatty acid (NEFA) from ~1.0 mM to ~0.2 mM, but concomitant infusion of intralipid during a clamp raised NEFA back to approximately fasting plasma concentrations (~1.2 mM) (Fig. S2F). Providing NEFA in competition with glucose led to significant reductions (~50%) in relative glucose oxidation in both soleus and quadriceps as measured by $V_{PDH}/V_{CS}$ (Fig. 3C-D), which occurred independently of changes in phosphorylation of PDH (Fig. S2H). Interestingly, infusion of intralipid increased intramyocellular acetyl-CoA and glutamate concentrations but not glucose-6-phosphate or citrate concentrations (Fig. 3F). This constellation of changes in intracellular metabolites with increased fat oxidation is consistent with acetyl-CoA-mediated allosteric inhibition of PDH activity, but not with Randle’s proposed mechanism of fat oxidation-induced inhibition of key glycolytic steps and glucose uptake, which predicts increases in glucose-6-phosphate and citrate concentrations with increased fat oxidation (25,51). Furthermore, we found that the significant
reduction in muscle glucose oxidation seen in the intralipid group was not accompanied by any changes in measures of *in vivo* muscle insulin response such as whole-body glucose uptake, muscle glucose transport, and muscle AKT phosphorylation (Fig. 3A-B, E), dissociating alterations in substrate oxidation from skeletal muscle insulin sensitivity.
Figure 1

A) Muscle Glucose Uptake

B) 2-Deoxyglucose Transport

C) Soleus

D) Quad

E) Soleus

F) Quad

For all figures, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001
Figure 2

A

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B

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C

PDH | CPT1b | CrAT | GAPDH

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D

Membrane PKCθ/Cytosolic PKCθ

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E

IRS-1 Associated PI3K Activity (Arbitrary Unit)

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F

pAKT/AKT (s473)

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Figure 3

A

Muscle Glucose Uptake (mg/(kg*min))

Soleus

V_{PDH}/V_{CS}

Clamp Intralipid

***

Quad

V_{PDH}/V_{CS}

Clamp Intralipid

**

E

Clamp Intralipid

pAKT/AKT (s473) (Arbitrary Units)

Clamp Intralipid

F

Concentration (nmol/g)

Control Intralipid

G-6-P Pyruvate Acetyl CoA Malonyl CoA Citrate Glutamate

* P=0.11
Supplementary Figure 1

A

Body Weight (g)

Chow | HFD

C

Fasting NEFA (mM)

Chow | HFD

E

Clamp Plasma Glucose (mg/dL)

Chow | HFD

G

Glucose Infusion Rate [mg/(kg.min)]

Chow | HFD

B

Fasting Plasma Glucose (mg/dL)

Chow | HFD

D

Fasting Plasma Insulin (µU/mL)

Chow | HFD

F

Clamp Plasma Insulin (µU/mL)

Chow | HFD

H

Basal

Clamp

pIRK/IRK (Y1162)

Chow | HFD
Supplementary Figure 2

A

Body Weight (g)

Clamp Intralipid

B

Fasting Plasma Glucose (mg/dL)

Clamp Intralipid

C

Fasting Plasm insulin (U/mL)

Clamp Intralipid

D

Clamp Plasma Glucose (mg/dL)

Clamp Intralipid

E

Clamp Plasma Insulin (U/mL)

Clamp Intralipid

F

NEFA (mM)

Basal Clamp Intralipid

G

pIRK/IRK (Y1162)

Clamp Intralipid

H

pPDH/PDH (Arbitrary Units)

Control Intralipid

pPDH (s293) pPDH (s232) pPDH (s300) PDH

Control Intralipid

s293 s232 s300
Supplementary Figure 3

A

R²=0.95
y = 1.023x + 0.997
P < 0.0001

B

R²=0.79
y = 0.958x - 0.263
P < 0.0001

- Regular chow (fasting)
- High fat diet (fasting)
- Regular chow (clamp)
- High fat diet (clamp)
- Regular chow (clamp + lipid infusion)
Discussion

The precise mechanism leading to the development of muscle insulin resistance has not yet been defined, but alterations in mitochondrial substrate preference has emerged as an attractive possibility. Randle was the first to hypothesize a path by which altered muscle substrate use, namely, increased muscle mitochondrial fatty acid oxidation, could inhibit not only glucose oxidation but glucose uptake in response to insulin (25,51). More recently as an expansion or update of Randle’s proposals, others have suggested that muscle insulin resistance is a result of ‘metabolic inflexibility,’ or muscle mitochondrial dysfunction that results in impairments in its ability to make smooth transitions between utilizing glucose or fatty acids as substrates depending on varying physiological needs. These hypotheses have located mitochondrial fuel preference as the nexus of muscle insulin resistance, in contrast to competing hypotheses that centered on defects in insulin signaling and insulin-mediated glucose transport. ‘Metabolic inflexibility’ necessarily entails two predictions about insulin resistant muscle: first, that fasting or basal substrate preference is altered in insulin resistant muscle due to mitochondrial dysfunction; and second, that insulin resistant muscle cannot increase glucose oxidation beyond its basal rate as a result of this mitochondrial dysfunction (26,27). In this hypothesis, the accumulation of intramyocellular lipid observed in insulin resistant muscle is hypothesized to be secondary to an impaired mitochondrial ability to oxidize fat, in opposition to other hypotheses that have proposed that the buildup of certain lipid metabolites (diacylglycerols, ceramides, etc.) lead to inhibition of insulin signaling.
While ‘metabolic inflexibility’ has become a widespread concept, the original studies by Kelley had significant methodological shortcomings that leave the precise relationship between muscle substrate oxidation and muscle insulin response hitherto unresolved (26,27). Furthermore, subsequent studies by other groups to identify possible candidate defects for muscle ‘metabolic inflexibility’ have predominantly relied on in vitro models or genetic manipulations to show altered mitochondrial substrate preference and oxidation. In this study, we used HFD-fed rats who were at the early stages of muscle insulin resistance to characterize early myocellular changes that mechanismically contributed to the development of this phenomenon. Importantly, by infusing a stable isotope tracer ([13C6]glucose) and analyzing enrichments in particular isotopologues of intramyocellular metabolites by LC-MS/MS, we were able to obtain a sensitive measure for muscle-specific glucose oxidation relative to total mitochondrial oxidation and relate this measure to in vivo indices of muscle insulin resistance in normal and HFD-fed rats as well as insulin sensitive and insulin resistant humans. With these methods, we sought to adjudicate between two competing hypotheses for the pathogenesis of muscle insulin resistance: one in which mitochondrial derangements lead to altered substrate preference and prevent insulin-mediated glucose uptake by a backlog mechanism; and two in which excess ectopic lipid build-up leads to interference with insulin signaling and prevents insulin-mediated glucose transport.

Our results contradict the predictions of the ‘metabolic inflexibility’ hypothesis in three major ways. First, while Kelley et al. had observed relative
glucose oxidation in normal fasting muscle contributed a significant proportion of total oxidation at 40% (27), we found relative glucose oxidation normal fasting muscle to be minimal at less than 10% in both soleus (primarily type I oxidative fibers) and quadriceps (primarily type II glycolytic fibers) (52). Second, while the ‘metabolic inflexibility’ hypothesis predicts that fasting substrate use in insulin resistant muscle would be significantly altered in favor of increased glucose oxidation, we found that the fasting muscles of insulin resistant rats did not differ from muscles of normal rats in their substrate preference and also relied overwhelmingly on fat oxidation (Fig. 1C-F). This lack of altered fasting substrate preference was corroborated by the absence of significant changes in muscle mitochondrial oxidative function and capacity in insulin resistant rats, as indicated by unchanged expressions of key mitochondrial oxidative enzymes such as PDH, CPT1b, and CrAT (Fig. 2C), and by unchanged concentrations of mitochondrial TCA cycle metabolites (Fig. 2B). Finally, whereas Kelley et al. had observed that insulin resistant muscle could not upregulate relative glucose oxidation in response to insulin (i.e. were metabolically inflexible), we found that muscles of insulin resistant rats increased relative glucose oxidation with insulin (i.e. were metabolically flexible), though the increase was blunted compared to normal muscle (Fig. 1C-F). This blunting of increased glucose oxidation was likely secondary to defects in insulin signaling (Fig. 2E) and insulin-mediated glucose transport (Fig. 2B, D, F) in insulin resistant rats. Taken together these data demonstrate that the muscles of insulin resistant rats were in fact metabolically
flexible but that this metabolic flexibility was constrained by defects in insulin-mediated glucose transport.

If muscle insulin resistance is not characterized by alterations in muscle substrate use, we asked conversely whether modulations in muscle substrate use in normal rats could affect insulin sensitivity. We found that changes in muscle substrate oxidation were dissociated from muscle insulin response. Infusion of intralipid in normal rats during a hyperinsulinemic-euglycemic clamp raised plasma fatty acid levels and significantly reduced glucose oxidation in muscle, but did not alter muscle glucose uptake, muscle glucose transport, or muscle insulin signaling (Fig. 3A-E). Furthermore, we did not find evidence for Randle’s proposed glucose-fatty acid cycle in which increased fatty acid oxidation in the myocyte could inhibit glucose oxidation and glucose uptake through inhibition of key regulated steps in glycolysis. While Randle had observed in in vitro studies that increased fat oxidation led to increased levels of acetyl CoA, citrate, and glucose-6-phosphate, we found that only acetyl CoA concentrations were increased in vivo (Fig. 3F), suggesting that fatty acid oxidation could inhibit glucose oxidation through allosteric inhibition of PDH (and not through inhibition of key glycolytic steps) but that this reduced PDH flux was ultimately inconsequential in the larger picture of muscle insulin response. Together with a recent study which found the complementary result that acutely increasing PDH flux with dichloroacetate did not affect muscle insulin sensitivity (53), these data dissociate changes in substrate oxidation from muscle insulin sensitivity and suggest that attempts to develop
therapeutics to treat insulin resistance and type 2 diabetes by targeting mitochondrial substrate preference would likely prove ineffective.

Though we do not find evidence of ‘metabolic inflexibility’ in the pathogenesis of muscle insulin resistance nor of any mechanistic relationship between modulation of mitochondrial substrate preference and muscle insulin sensitivity, these data should not be taken as evidence that mitochondrial dysfunction more broadly is insignificant to the development of metabolic disease. In fact, robust evidence exists that reductions in mitochondrial function may be an important predisposing contributor to myocellular lipid accumulation and muscle insulin resistance in the lean offspring of parent with type 2 diabetics (54–56) and the elderly (57). These effects, however, could be attributed to reductions in total mitochondrial energy expenditure leading to increased ectopic lipid accumulation and not to specific alterations in substrate utilization. Consistent with overall reductions in skeletal muscle mitochondrial function as previously documented by both in vivo $^{31}$P magnetic resonance spectroscopy (MRS) (56) and $^{13}$C MRS (54) we also observed a reduction in insulin-stimulated muscle mitochondrial $V_{CS}$ flux in the insulin resistant subjects as assessed by in vivo $^{13}$C MRS. Thus, it is important to distinguish mitochondrial substrate preference per se, which these data demonstrate has little impact on the development of muscle insulin resistance, from reductions in overall mitochondrial function and fat oxidation that leads to the imbalance of energy intake and expenditure, ectopic lipid build-up, and insulin resistance. In support of this hypothesis Knowles et al. have recently identified a gene variant in N-acetyl transferase-2 (Nat2) in humans that is associated with
T2DM (58). Nat1 (mouse homologue) knockout mice display reduced whole-body energy metabolism (58,59), reduced mitochondrial function, increased ectopic lipid deposition and liver and muscle insulin resistance (59).

In summary, these studies demonstrate that in contrast to the tenets of the metabolic flexibility hypothesis there are no alterations in basal rates of mitochondrial $V_{PDH}/V_{CS}$ flux in insulin resistant human or rodent skeletal muscle thus providing strong evidence against hypotheses that relate alterations in muscle substrate preference to muscle insulin resistance.
References


