

Mitochondrial Substrate Availability and Its Role in Lipid-Induced Insulin Resistance and Proinflammatory Signaling in Skeletal Muscle

Christopher Lipina,¹ Katherine Macrae,¹ Tamara Suhm,² Cora Weigert,^{2,3} Agnieszka Blachnio-Zabielska,⁴ Marcin Baranowski,⁴ Jan Gorski,⁴ Karl Burgess,⁵ and Harinder S. Hundal¹

The relationship between glucose and lipid metabolism has been of significant interest in understanding the pathogenesis of obesity-induced insulin resistance. To gain insight into this metabolic paradigm, we explored the potential interplay between cellular glucose flux and lipid-induced metabolic dysfunction within skeletal muscle. Here, we show that palmitate (PA)-induced insulin resistance and proinflammation in muscle cells, which is associated with reduced mitochondrial integrity and oxidative capacity, can be attenuated under conditions of glucose withdrawal or glycolytic inhibition using 2-deoxyglucose (2DG). Importantly, these glucopenic-driven improvements coincide with the preservation of mitochondrial function and are dependent on PA oxidation, which becomes markedly enhanced in the absence of glucose. Intriguingly, despite its ability to upregulate mitochondrial PA oxidation, glucose withdrawal did not attenuate PA-induced increases in total intramyocellular diacylglycerol and ceramide. Furthermore, consistent with our findings in cultured muscle cells, we also report enhanced insulin sensitivity and reduced proinflammatory tone in soleus muscle from obese Zucker rats fed a 2DG-supplemented diet. Notably, this improved metabolic status after 2DG dietary intervention is associated with markedly reduced plasma free fatty acids. Collectively, our data highlight the key role that mitochondrial substrate availability plays in lipid-induced metabolic dysregulation both *in vitro* and *in vivo*. *Diabetes* 62:3426–3436, 2013

There is significant evidence in the literature linking elevation in free fatty acid (FFA) levels with development of peripheral insulin resistance and its associated metabolic perturbations, including impaired glucose uptake (1). However, as yet, there is no unified view that adequately explains how these pathophysiological events develop. Randle et al. (2) first postulated that increased mitochondrial fatty acid oxidation (associated with FFA oversupply) may impair glucose uptake and utilization via metabolite-induced allosteric

inhibition of key glycolytic enzymes. Specifically, inhibition of pyruvate dehydrogenase and phosphofructokinase by acetyl CoA and citrate generated from excessive FFA oxidation would culminate in accumulation of glucose-6-phosphate (G6P) that, in turn, would promote allosteric inhibition of hexokinase with a concomitant reduction in glucose uptake. However, the concept that suppressed glycolytic flux underlies lipid-induced metabolic dysfunction has been challenged by an alternative school of thought implicating ectopic accumulation of lipids as the primary cause of insulin resistance in skeletal muscle (3,4). In particular, lipid oversupply in the form of excess saturated fatty acids including palmitate (PA) (C16:0) promotes intramyocellular accumulation of bioactive lipids such as diacylglycerol (DAG) and ceramide. These lipids have been implicated strongly in the development of insulin resistance via their inhibitory effects on proximal insulin signaling, and as such, their effects may depend or be further accentuated by glucose overloading (i.e., glucolipotoxicity). Notably, activated DAG-sensitive protein kinase C (PKC) isoforms suppress insulin action via elevated serine phosphorylation of insulin receptor substrates, such as insulin receptor substrate (IRS)-1 (5,6), whereas ceramide induces a targeted loss in activation of the serine/threonine kinase, Akt, by insulin (7). Both IRS and Akt serve as critical components of the insulin signaling cascade, and so their dysregulation impairs key processes under the control of insulin, including glucose uptake and glycogen synthesis.

In accordance with evidence supporting a role for lipotoxic intermediates in the pathogenesis of insulin resistance, a number of studies have also explored the relationship between insulin sensitivity and mitochondrial function. Importantly, various indicators of impaired mitochondrial capacity have been reported in skeletal muscle from insulin-resistant or obese human subjects including, for example, a reduction in the activity or expression of carnitine palmitoyltransferase-1, which mediates mitochondrial fatty acid uptake, and that of various oxidative enzymes and respiratory chain components coinciding with suppressed ATP synthesis (8–13). Furthermore, obesity-induced reductions in peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α , a key transcriptional coordinator of mitochondrial biogenesis, and elevated expression of muscle nuclear factor κ B (NF- κ B)-dependent proinflammatory genes (e.g., interleukin-6 [IL-6], tumor necrosis factor- α), may contribute significantly to FFA-induced mitochondrial dysfunction and insulin resistance (12,14–18). Collectively, these observations support the idea that mitochondrial dysfunction (associated with lipid oversupply) may restrict FFA oxidation and promote their greater partitioning into bioactive lipid intermediates, such as ceramide and DAG, that negatively regulate insulin action.

From the ¹Division of Cell Signalling and Immunology, Sir James Black Centre, College of Life Sciences, University of Dundee, Dundee, U.K.; the ²Division of Endocrinology, Diabetology, Angiology, Nephrology, Pathobiochemistry and Clinical Chemistry, Department of Internal Medicine, University of Tübingen, Tübingen, Germany; the ³Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Centre Munich at the University of Tübingen, Tübingen, Germany (Paul Langerhans Institute Tübingen), Member of the German Centre for Diabetes Research; the ⁴Department of Physiology, Medical University of Białystok, Białystok, Poland; and the ⁵Glasgow Polyomics Metabolomics Facility, University of Glasgow, Glasgow, U.K.

Corresponding author: Harinder S. Hundal, h.s.hundal@dundee.ac.uk.

Received 15 February 2013 and accepted 29 May 2013.

DOI: 10.2337/db13-0264

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-0264/-/DC1>.

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Herein, given the current focus on fuel substrate-derived metabolites, lipotoxic intermediates, and mitochondrial energetics, we sought to explore their interrelationship and involvement in obesity and FFA-induced metabolic dysregulation by assessing the effects of restricting glucose utilization both *in vitro* and *in vivo*. Together, our data underscore the important role that mitochondrial substrate overload, in particular with respect to glucose and fatty acid provision, plays in the development of lipid-mediated insulin resistance and associated proinflammatory cytokine production in skeletal muscle.

RESEARCH DESIGN AND METHODS

Ten-week-old male obese (*fa/fa*) and lean (*Fa/fa*) Zucker rats obtained from Harlan UK (Oxon, U.K.) were maintained on a 12-h light/dark cycle and fed *ad libitum* a standard or 2-deoxyglucose (2DG) (0.4% w/w) supplemented chow diet for 28 days. The 2DG diet was prepared by pulverizing standard chow pellets and reconstituting with 2DG powder. Body weights and food intake were monitored twice weekly. After the 28-day dietary regimen, animals were fasted overnight (16 h) and subsequently anesthetized by injection of sodium pentobarbitone (60 mg/kg *i.p.*). Blood sampling, careful excision, and rapid freezing in liquid nitrogen of soleus from one hindlimb were then performed prior to administration of insulin (10 units/kg *i.v.*, Actrapid; Novo Nordisk, Bagsvaerd, Denmark). Five minutes post-insulin administration, soleus muscle from the contralateral hindlimb was removed and immediately frozen in liquid nitrogen for storage at -80°C . All animal procedures complied with the U.K. Animal Scientific Procedures Act.

Muscle cell culture, cell treatments, and analysis. Methods used for culturing and treating rat (L6) and primary human myotubes with PA and their preparation for immunoblotting, RNA extraction, conventional RT-PCR and real-time quantitative (qPCR) analysis, PA oxidation, and whole-cell oxygen consumption and analysis of intramyocellular DAG and ceramide have previously been described (19–22) or are described in the Supplementary Data.

Measurement of intracellular metabolites. L6 myotubes were incubated with 0.5 mmol/L PA in the absence or presence of 5 mmol/L glucose as indicated in the appropriate figure legends. After treatment, cells were lysed in ice-cold chloroform/methanol/water (1:3:1) and resulting samples analyzed by liquid chromatography–mass spectrometry (LCMS) as previously described (23). Analysis of LCMS data was performed using MzMatch (24) and IDEOM (23) software, with metabolites confirmed by mass and retention time analysis being matched against authentic standards or classified as putative, based on mass, predicted retention time, and biochemical reference (23).

Determination of blood and tissue biochemistry. Plasma FFA concentration was measured using the EnzyChrom Free Fatty Acid Assay Kit from BioAssay Systems (Hayward, CA). Triglyceride concentration in blood plasma was determined using the Triglyceride Assay kit from Cayman Chemical (Ann Arbor, MI). Circulating blood glucose levels were measured using an Alpha-TRAK Blood Glucose Monitoring System (Abbott Laboratories).

Statistical analyses. For multiple comparisons, statistical analysis was performed using one-way ANOVA or a *t* test where appropriate. Data analysis was performed using GraphPad Prism software and considered statistically significant at *P* values <0.05 .

RESULTS

Effects of PA and glucose availability on Akt-directed insulin signaling. Sustained (16 h) PA overprovision caused a substantial reduction in the insulin-dependent phosphorylation/activation of Akt and that of forkhead box O3 (FOXO3a) and glycogen synthase kinase-3 α/β ; both established Akt targets in L6 myotubes (Fig. 1A). To determine whether this reduced insulin signaling capacity was a consequence of substrate competition, we investigated the effects of incubating myotubes with PA for 16 h in 1) glucose-free media, 2) glucose-free media to which 2DG (a nonmetabolizable glucose analog and glycolytic inhibitor) was added as a glucose substitute, or 3) glucose-containing media to which 2DG was added as a competitive glucose supplement. This strategy revealed that while Akt phosphorylation by insulin was severely blunted by PA when glucose was present (Fig. 1B [compare

lanes 1 and 2]), this inhibition was not detected in myotubes incubated with PA in the absence of glucose or when glucose had been substituted or media cosupplemented with 2DG (Fig. 1B [compare lanes 4 and 8 with 2]). In line with the ability to restrain the insulin-desensitizing effect of PA upon glucose withdrawal or 2DG provision, phosphorylation of FOXO3a and glycogen synthase kinase-3 α/β was also enhanced (Fig. 1B). This enhancement was not just restricted to proximal insulin signaling events but was also evident by changes in hexokinase II gene expression, which represents a more distal insulin-regulated response (Fig. 1C). Since the insulin-desensitizing effects of PA on Akt-directed insulin signaling were potently repressed in the absence of glucose, we assessed whether we could define a threshold glucose concentration at which glucolipototoxicity was no longer evident. Figure 1D shows that 0.5 mmol/L PA induced a significant reduction in insulin-dependent Akt phosphorylation when cells were incubated in the presence of glucose at concentrations >0.2 mmol/L.

To exclude the possibility that the insulin-sensitizing effect conferred by limiting glucose availability/metabolism may be a phenomenon restricted to rat skeletal muscle cells, Fig. 1E shows that primary human skeletal myotubes exposed to PA also exhibit significant reduction in Akt phosphorylation, which was antagonized by glucose deprivation.

It is important to stress that the reduction in Akt phosphorylation that we observe in muscle cells after incubation with PA (Fig. 1) is not restricted to this fatty acid alone. Indeed, exposure of L6 myotubes to stearate (a C18:0 saturated fatty acid) or to a fatty acid mixture comprising PA, stearate, oleate, and palmitoleate (at concentrations found in plasma of obese, insulin resistant rodents) promotes a loss in insulin-dependent Akt phosphorylation, which, in both instances, can be attenuated if muscle cells are held in glucose-free or 2DG-supplemented media (Supplementary Fig. 1).

Effect of PA and glucose availability on proinflammatory signaling. Sustained myotube exposure to PA also stimulates NF- κ B signaling, which depends critically upon upstream activation of the mitogen-activated protein kinase (extracellular signal-related kinase [ERK]) pathway (19). Figure 2A shows that PA induces a dose-dependent activation of ERK1/2 and I κ B kinase (IKK) α/β with concomitant loss of inhibitor of κ B (I κ B) α . However, this PA-induced activation of ERK and I κ B α loss were attenuated in myotubes maintained in glucose-free media (Fig. 2B and C) or when either glucose was substituted with 2DG or this glycolytic inhibitor was added to glucose-containing media (Fig. 2C [compare lanes 6 and 8 with lane 2]). However, unlike Akt activation (Fig. 1C), the loss of this protein in response to PA was only abrogated when glucose was completely absent from the media. Consistent with the stimulation that PA promotes in NF- κ B signaling when glucose is available, PA induced robust increases in the expression of two NF- κ B target genes: IL-6 and cytokine-induced neutrophil chemoattractant-1 (CINC-1) (the rat homolog of IL-8), which was not seen in myotubes deprived of glucose (Fig. 2E and F). These findings were replicated in primary human myotubes (Fig. 2G).

Effects of PA and glucose availability upon intramyocellular DAG and ceramides. PA-induced increases in intramyocellular DAG and ceramide have been shown to impair IRS-1 and Akt activation (6,25,26). The ability to restrain the insulin-desensitizing effect of PA when glucose is limiting may be linked to a reduction in PA-driven

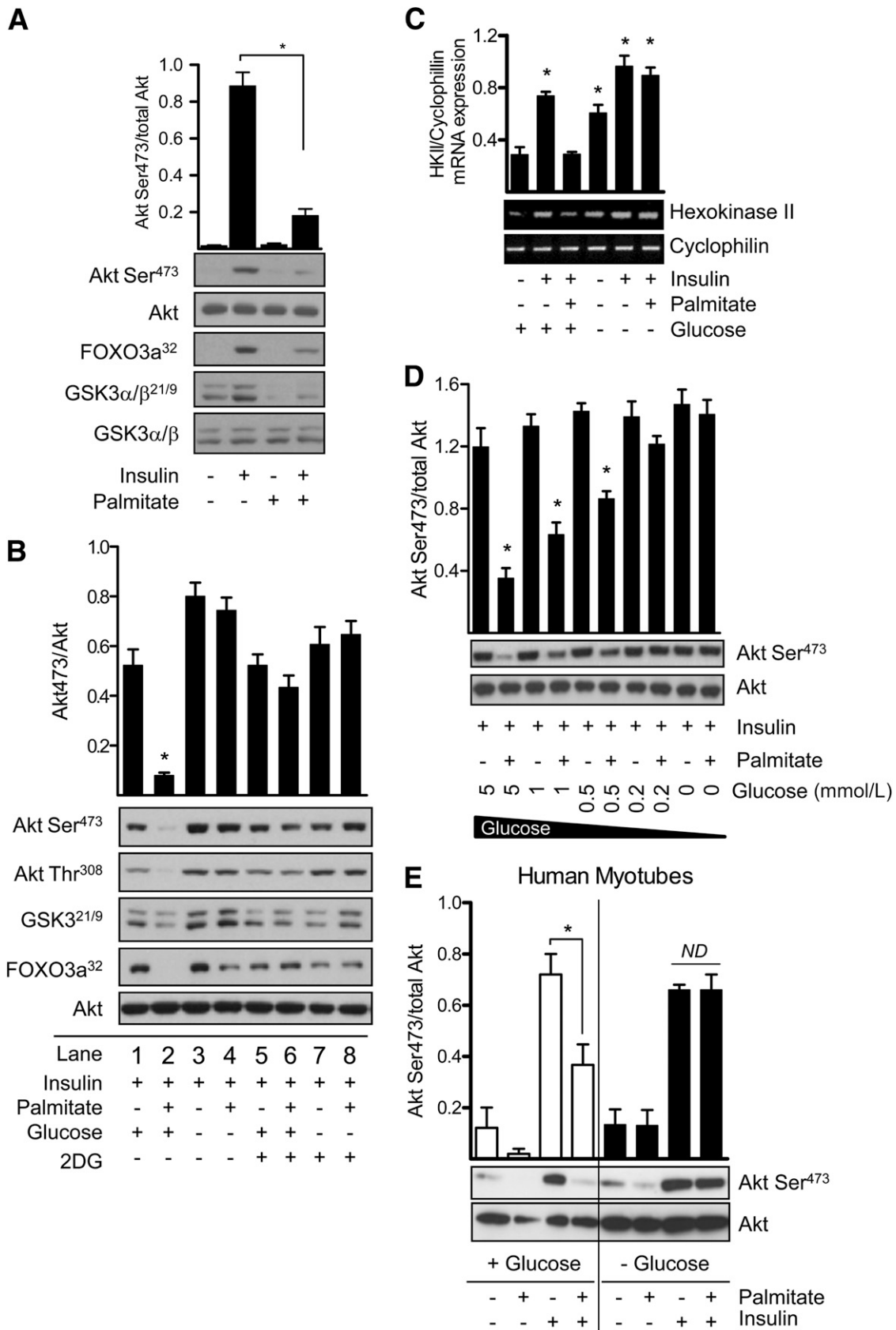


FIG. 1. Limiting glucose availability or its metabolism antagonizes PA-induced insulin resistance in L6 myotubes. **A** and **B**: L6 myotubes were treated with 0.5 mmol/L PA for 16 h in the absence or presence of 5 mmol/L D-glucose with or without 5 mmol/L 2DG as indicated prior to stimulation with insulin (20 nmol/L for 10 min). Resulting cell lysates were analyzed by immunoblotting using the antibodies indicated. **P* < 0.05 between bars indicated or vs. non-PA treated. Data values presented as mean ± SEM, *n* = 3. **C**: L6 myotubes were incubated with 0.5 mmol/L PA in the absence or presence of 5 mmol/L glucose for a total of 16 h. For the final 4 h, cells were treated with 100 nmol/L insulin or vehicle control prior to extraction of total cellular RNA. Hexokinase II (HKII) mRNA expression relative to the internal control cyclophilin was subsequently

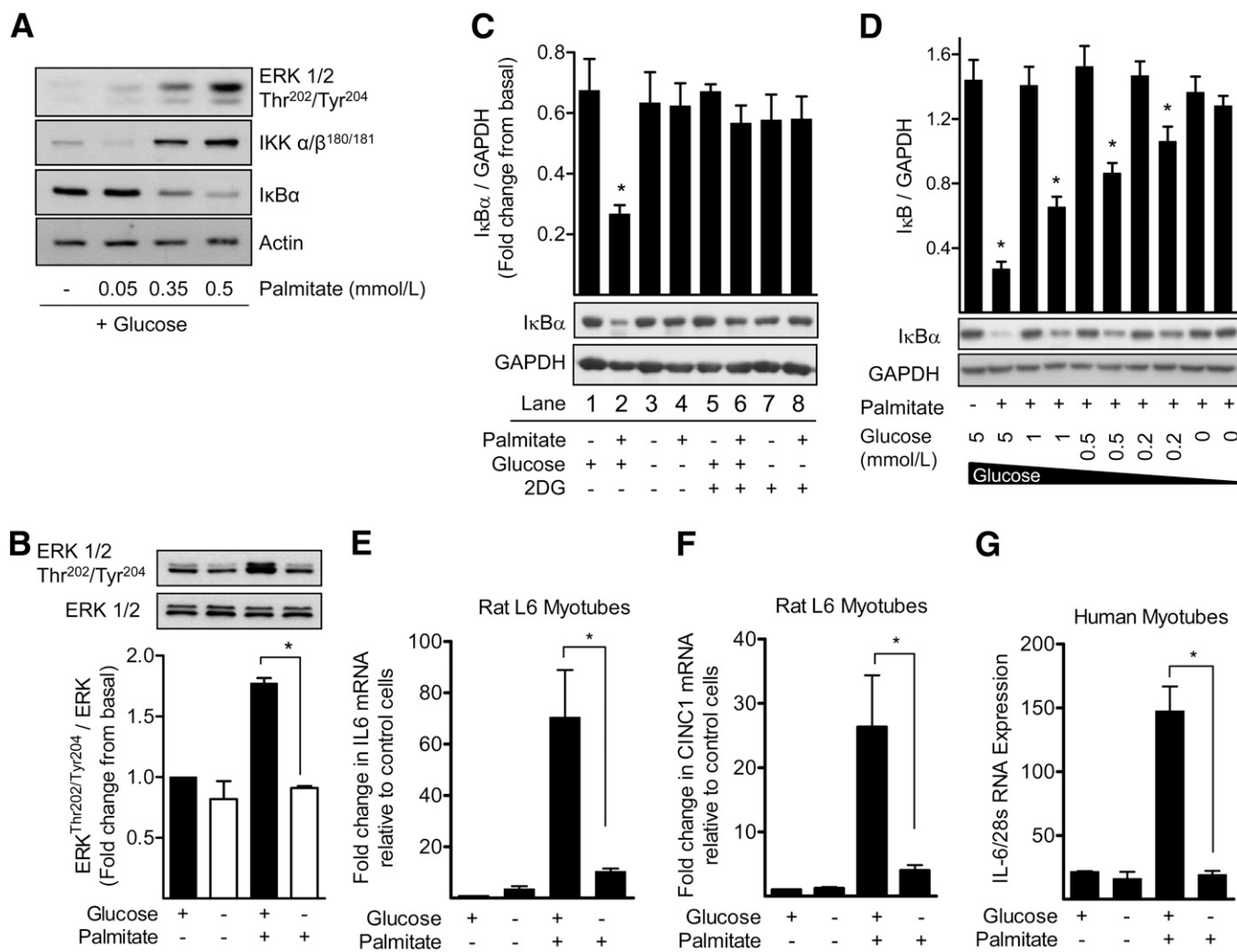


FIG. 2. Limiting glucose availability or its utilization antagonizes PA-induced inflammation in L6 myotubes. *A–C*: L6 myotubes were treated with different concentrations of PA (*A*) or 0.5 mmol/L PA (*B* and *C*) for 16 h in media containing or lacking either 5 mmol/L D-glucose or 5 mmol/L 2DG as indicated. Resulting cell lysates were immunoblotted using the antibodies indicated. *D*: L6 myotubes were incubated with 0.5 mmol/L PA for 16 h in the presence of different concentrations of glucose as indicated. Resulting cell lysates were immunoblotted using the antibodies shown. Relative band intensity values presented are the mean \pm SEM from three independent experiments. *Significant change ($P < 0.05$) from the untreated control (*lane 1*). *E–G*: L6 myotubes (*E* and *F*) and human primary skeletal muscle myotubes (*G*) were treated with 0.5 mmol/L (for 16 h) or 0.25 mmol/L (for 22 h) PA, respectively, in the absence or presence of 5 mmol/L D-glucose. After cell treatments, total RNA was extracted and used to determine relative mRNA expression of IL-6 and CINC-1 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 28S rRNA by qPCR as indicated. Data values presented are mean \pm SEM ($n = 3$ for L6 myotubes; $n = 4$ for human primary skeletal muscle myotubes). * $P < 0.05$ between the indicated bars.

accumulation of DAG and ceramides. However, Fig. 3*A* and *B* shows that while DAG and ceramides were increased by more than twofold when myotubes were incubated with PA in glucose-containing media, glucose withdrawal or its substitution with 2DG had no impact on the accumulation of either lipid. Our liquid chromatography–mass spectrometry analysis permits quantification of multiple DAGs and ceramides and reveals that while most are elevated in response to PA, glucose withdrawal or glycolytic inhibition with 2-DG only affected accumulation of a few select DAG and ceramide species (Supplementary Fig. 2 [DAGs:

C18:0/C18:0, C18:1/C18:1] and Supplementary Fig. 3 [ceramide: C14:0, C18:1]). It is possible that under these circumstances, the lipotoxic effects associated with DAG and ceramide are negated by their generation in membrane compartments spatially segregated from those in which insulin signaling is initiated. To test this possibility, we incubated myotubes with increasing concentrations of C2 ceramide, a cell-permeable ceramide analog whose cellular distribution is unlikely to be constrained to specific membrane pools. Figure 3*C* shows that regardless of glucose availability, Akt activation by insulin was inhibited by C2 ceramide.

determined by RT-PCR analysis. Relative band intensity values presented are the mean \pm SEM from three independent experiments. *Significant change ($P < 0.05$) from the untreated control (*lane 1*). *D*: L6 myotubes were incubated with 0.5 mmol/L PA for 16 h in the presence of different concentrations of glucose as indicated prior to stimulation with insulin (20 nmol/L for 10 min). Resulting cell lysates were immunoblotted using the antibodies shown. Relative band intensity values presented are the mean \pm SEM from three independent experiments. *Significant change ($P < 0.05$) from the insulin/5 mmol/L glucose bar value (*lane 1*). *E*: Representative immunoblot for total and phosphorylated Ser⁴⁷³ Akt in primary human skeletal muscle myotubes treated with 0.25 mmol/L PA for 22 h in media containing or lacking 5 mmol/L D-glucose prior to stimulation with insulin (100 nmol/L for 10 min). Data values presented as mean \pm SEM, $n = 4$. (* $P < 0.05$ between the indicated bars.) ND, no difference.

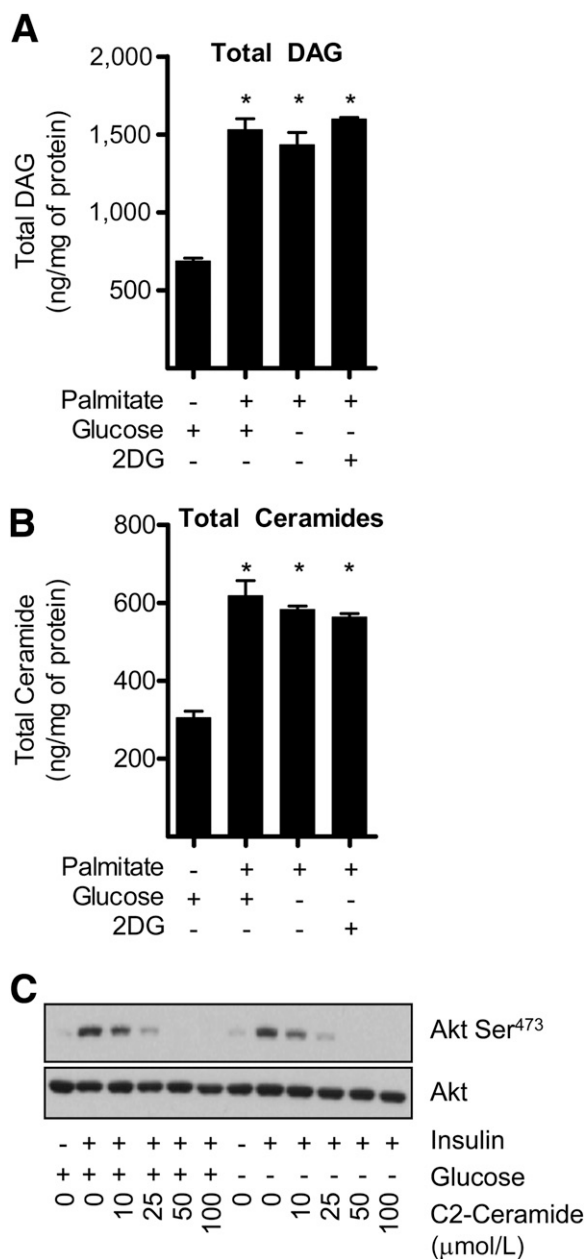


FIG. 3. Limiting glucose availability does not prevent PA-induced ceramide and DAG accumulation in L6 myotubes. **A** and **B**: Total cellular DAG (**A**) and ceramide (**B**) content in L6 myotubes after treatment with 0.5 mmol/L PA (or vehicle control) for 16 h in the absence or presence of 5 mmol/L D-glucose or 5 mmol/L 2DG as indicated. **C**: L6 myotubes were treated with different concentrations of C2 ceramide for 2 h in serum-free media containing or lacking 5 mmol/L D-glucose prior to insulin stimulation (20 nmol/L for 10 min). Resulting cell lysates were immunoblotted for total and phosphorylated Ser⁴⁷³ Akt. Data are presented as mean \pm SEM from three independent experiments.

Effects of substrate availability on mitochondrial function. Insulin resistance and heightened proinflammatory signaling may be a consequence of mitochondrial dysfunction induced by substrate overload. Figure 4A shows that cellular respiration fell significantly ($\sim 50\%$) when myotubes were incubated with PA and glucose. This reduced aerobic capacity was not observed when cells were exposed to PA alone or when glucose was substituted with 2DG. In line with these observations, analysis of ATP and ADP revealed that the ATP-to-ADP ratio fell significantly from 16.2 ± 1.2 to 8.1 ± 1.0 in myotubes incubated with

PA and glucose, whereas this ratio was unaltered in cells exposed to PA alone (not shown). The ability to maintain cellular respiration in myotubes treated with just PA implies that cells may oxidize PA more efficiently. Indeed, PA oxidation was approximately threefold greater than in myotubes incubated with PA and glucose together (Fig. 4B). This increase in PA oxidation was sensitive to 2-bromopalmitate and etomoxir, two carnitine palmitoyltransferase-1 inhibitors (27). To assess whether the reduced respiratory rate associated with PA and glucose overload is a consequence of impaired mitochondrial integrity/function, we monitored the abundance of PGC-1 α , cytochrome c oxidase subunit IV (COXIV), and succinate dehydrogenase (SDHA). These proteins were reduced substantially in cells treated with PA and glucose but maintained when either nutrient was present alone or when glucose metabolism was inhibited by 2DG (Fig. 4C).

Consistent with the reduction in SDHA associated with glucose/PA overload (Fig. 4C), we observed a significant decline in fumarate, the product of succinate oxidation by SDHA, and malate, which is generated from hydration of fumarate. Intriguingly, both metabolites were significantly elevated in myotubes incubated with PA alone, most likely due to increased anaerobic flux via reactions contributing to their maintenance. Since glucose withdrawal or glycolytic inhibition is protective against the insulin-desensitizing and proinflammatory effects of PA, we hypothesized that this may depend on using PA as fuel when glucose supply is restricted. Indeed, inhibiting mitochondrial PA uptake using 2-bromopalmitate induced a dose-dependent reduction in insulin-stimulated Akt phosphorylation and I κ B α loss in response to PA despite the absence of glucose (Fig. 4E).

The ability to sustain PA oxidation in the absence of glucose requires maintaining levels of key tricarboxylic acid (TCA) cycle metabolites such as oxaloacetate (OAA) via anaerobic reactions like that catalyzed by pyruvate carboxylase. As anticipated, cells incubated with PA alone exhibit a dramatic reduction in glycolytic intermediates, such as G6P and glyceraldehyde 3-phosphate (Fig. 5A). Intriguingly, however, despite withdrawing glucose the abundance of pyruvate (the end product of glycolysis) was maintained in myotubes treated with PA alone (Fig. 5B). We postulated that irreversible carboxylation of pyruvate by pyruvate carboxylase may help sustain mitochondrial anaerobiosis. To test this hypothesis, the effect of phenylacetic acid (PAC), a pyruvate carboxylase inhibitor, was assessed in myotubes treated with PA in the absence and presence of glucose. Figure 5C and D show that while the insulin-desensitizing and proinflammatory effect of PA is attenuated by glucose withdrawal, this protective effect was progressively eroded by PAC in a dose-dependent manner.

Since pyruvate would normally be generated via glycolysis, it is likely that in the absence of glucose it is generated by transamination of alanine prior to being channeled into mitochondria for OAA synthesis by pyruvate carboxylase. Figure 5E and F shows that aminooxyacetate, a transaminase inhibitor (28), antagonized the protective effect conferred by glucose withdrawal upon Akt phosphorylation and I κ B α .

Effects of 2DG on metabolic homeostasis in obese Zucker (*fa/fa*) rats. To validate whether restricting skeletal muscle glucose utilization confers similar insulin-sensitizing and anti-inflammatory effects in vivo, the effect of a glucopenic dietary intervention in an appropriate animal model was investigated. Obese male Zucker (*fa/fa*) rats and their lean littermates were placed on either a

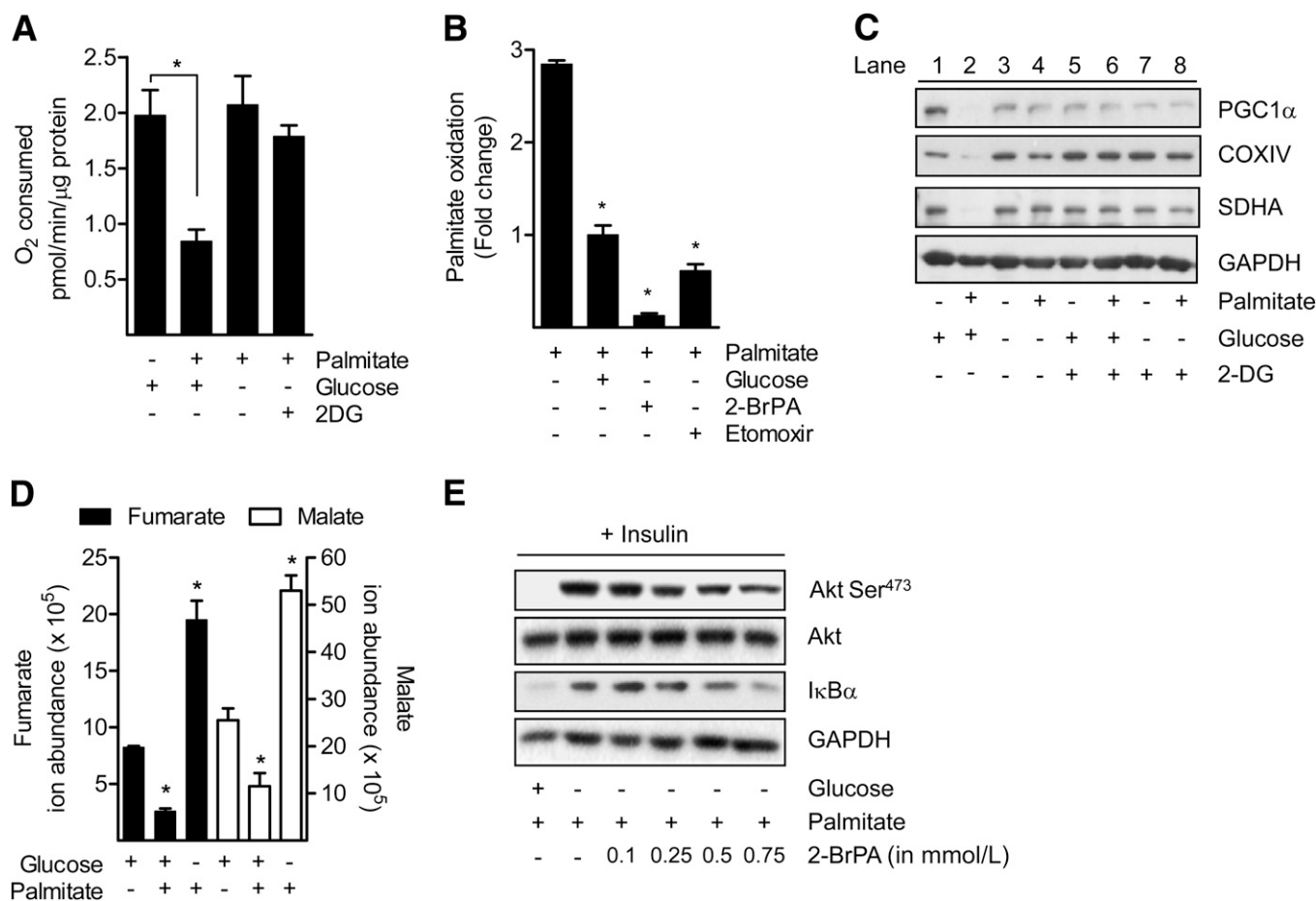


FIG. 4. Improvements in mitochondrial function in response to restricted glucose availability or its utilization contributes to enhanced insulin sensitivity and reduced proinflammatory signaling. **A:** L6 myotubes were incubated with 0.5 mmol/L PA (or vehicle control) for 16 h in media supplemented with or without 5 mmol/L D-glucose or 5 mmol/L 2DG as shown. Oxygen consumption rates were subsequently measured as described in Supplementary Data. * $P < 0.05$ vs. non-PA treated. **B:** L6 myotubes were incubated in the absence or presence of 5 mmol/L D-glucose for 3 h in the absence or presence of 2-bromopalmitate (2-BrPA) (100 μ mol/L) or etomoxir (100 μ mol/L), as indicated, prior to determining the rate of [³H]PA oxidation. * $P < 0.05$ compared with glucose-containing treatment. **C:** Representative immunoblot for PGC-1 α , COXIV, SDHA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in L6 myotubes treated with 0.5 mmol/L PA (or vehicle control) for 16 h in the absence or presence of 5 mmol/L D-glucose or 5 mmol/L 2DG. **D:** Fumarate and malate content in L6 myotubes after treatment with 0.5 mmol/L PA (or vehicle control) for 16 h in the absence or presence of 5 mmol/L D-glucose. * $P < 0.05$ vs. non-PA treated. **E:** L6 myotubes were incubated with 0.5 mmol/L PA (or vehicle control) for 16 h in media containing or lacking 5 mmol/L D-glucose in the presence of different concentrations of 2-bromopalmitate as indicated. Cells were then stimulated with insulin (20 nmol/L for 10 min) and resulting cell lysates used to immunoblot for native and phosphorylated Ser⁴⁷³ Akt, I κ B α , and glyceraldehyde-3-phosphate dehydrogenase. All data are presented as mean \pm SEM from three independent experiments.

normal chow diet or one supplemented with 2DG for 28 days. Figure 6A shows that lean and obese rats on the 2DG diet gain considerably less weight than their respective counterparts fed the normal chow diet, although the effect was far greater (approximately threefold) in obese than lean animals (Supplementary Table 1). Although weight gain in the lean animals on the 2DG diet was consistently lower at each measurement point over the 28-day period compared with lean controls fed the standard diet, the difference between these groups (unlike the obese animals) fell short of being significant. Despite no enforced restriction on how much food animals could eat, obese rats consumed marginally less on the 2DG diet, whereas no differences were observed in food intake in lean animals (Fig. 6B).

Effects of 2DG upon plasma glucose, FFAs, triglyceride, epididymal fat pad, and soleus mass. Figure 6C shows that fasting plasma glucose remained significantly higher in obese compared with lean animals reflecting the fact that the obese animals used in our study are severely glucose intolerant and insulin resistant. Intake of 2DG induced a

modest reduction in circulating glucose in both lean and obese rats (by \sim 18%). This finding is consistent with the known glucopenic effect induced by 2DG consumption (29). Obese Zucker rats are characteristically hyperlipidemic. In line with this, plasma triglyceride was elevated by \sim 11-fold and FFAs by \sim 2.5-fold compared with their lean littermates (Fig. 6D and E). Although 2DG intake induced a slight reduction in plasma triglyceride in obese animals, this was not significant (Fig. 6D). In contrast, 2DG consumption induced a profound reduction in plasma FFAs in obese rats (Fig. 6E), which occurs alongside a modest but significant fall in epididymal fat mass (Fig. 6F). Similar reductions were not apparent in lean animals fed 2DG. No notable changes in soleus mass between animals fed the standard chow or 2DG diets were observed (data not shown).

2DG intake improves insulin signaling and reduces inflammatory tone in muscle of obese rats. Figure 7A shows that there were no detectable differences in the insulin-dependent phosphorylation of Akt in solei from three lean animals fed either the standard or the 2DG diet.

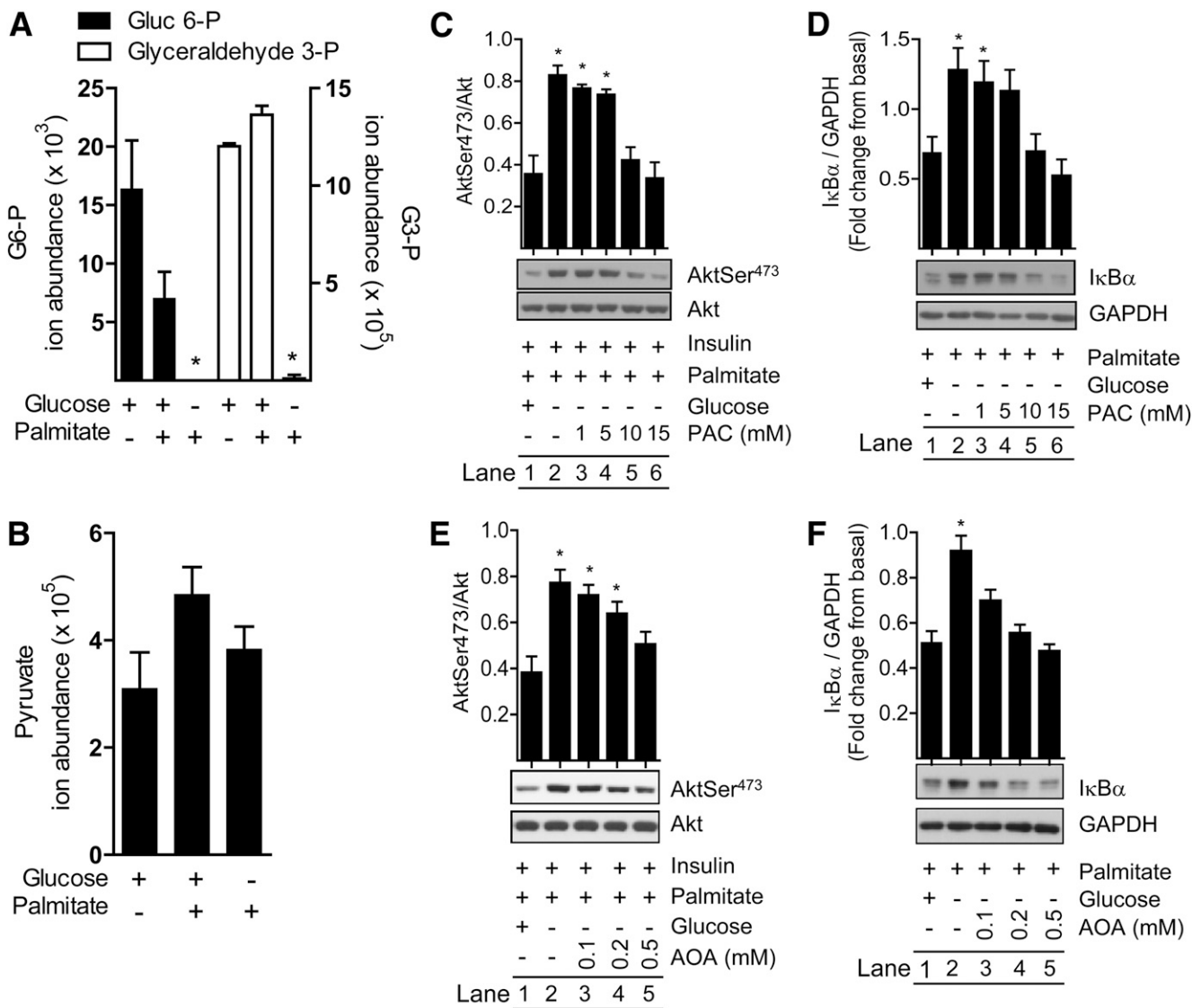


FIG. 5. Mitochondrial anaplerosis via sustained cellular pyruvate underpins the beneficial metabolic effects of glucose withdrawal. **A** and **B**: Intracellular levels of G6P (Gluc 6-P) and glyceraldehyde 3-phosphate (glyceraldehyde 3-P) (**A**) and pyruvate (**B**) in L6 myotubes treated with 0.5 mmol/L PA (or vehicle control) for 16 h in the absence or presence of 5 mmol/L D-glucose as determined by LCMS analysis. **C–F**: L6 myotubes were incubated with 0.5 mmol/L PA (or vehicle control) for 16 h in the absence or presence of 5 mmol/L D-glucose and different concentrations of PAC (**C** and **D**) or aminooxyacetic acid (AOA) (**E** and **F**) as shown. After treatments, resulting cell lysates were subjected to SDS-PAGE and immunoblotted for total and phosphorylated Ser⁴⁷³ Akt, IκBα, and GAPDH as indicated. All data are presented as mean ± SEM (n = 3). *P < 0.05 vs. cells treated with D-glucose alone (**A**). *P < 0.05 vs. PA-treated cells in the presence of 5 mmol/L D-glucose (**C–F**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

In contrast, a similar analysis from five obese animals on the standard diet revealed that muscle Akt phosphorylation in response to insulin was severely blunted, consistent with the significant insulin resistance exhibited by these animals (Fig. 7B). Strikingly, solei from five separate obese animals fed 2DG all exhibited a significant increase in Akt phosphorylation (Fig. 7B). Analysis of IκBα abundance revealed a significant fall in soleus of obese animals on the normal diet compared with those fed 2DG (Fig. 7C). In line with this, the relative expression of muscle IL-6 and CINC-1 mRNA was significantly lower in animals fed the 2DG-supplemented diet (Fig. 7D).

DISCUSSION

A central tenet of the Randle hypothesis is that increased provision and oxidation of FFAs in skeletal muscle

suppresses uptake and utilization of glucose as a consequence of an increase in G6P that results in allosteric inhibition of key glycolytic enzymes (2). Our findings do not support this premise, given that we observed a modest reduction in the steady-state abundance of G6P when muscle cells were rendered insulin resistant by PA in the presence of glucose (Fig. 5A). Lipid infusion and clamp studies also show that while raising plasma FFAs in healthy individuals impairs glucose handling in skeletal muscle, such disturbances are preceded by a fall rather than an increase in intramuscular G6P as originally hypothesized by Randle (30). Together, these observations suggest that mechanisms other than a reduction in glycolytic drive are likely to play a more prominent role in fatty acid-induced insulin resistance in skeletal muscle.

Numerous studies suggest that accumulation of fatty acid-derived DAG and ceramide are better predictors of

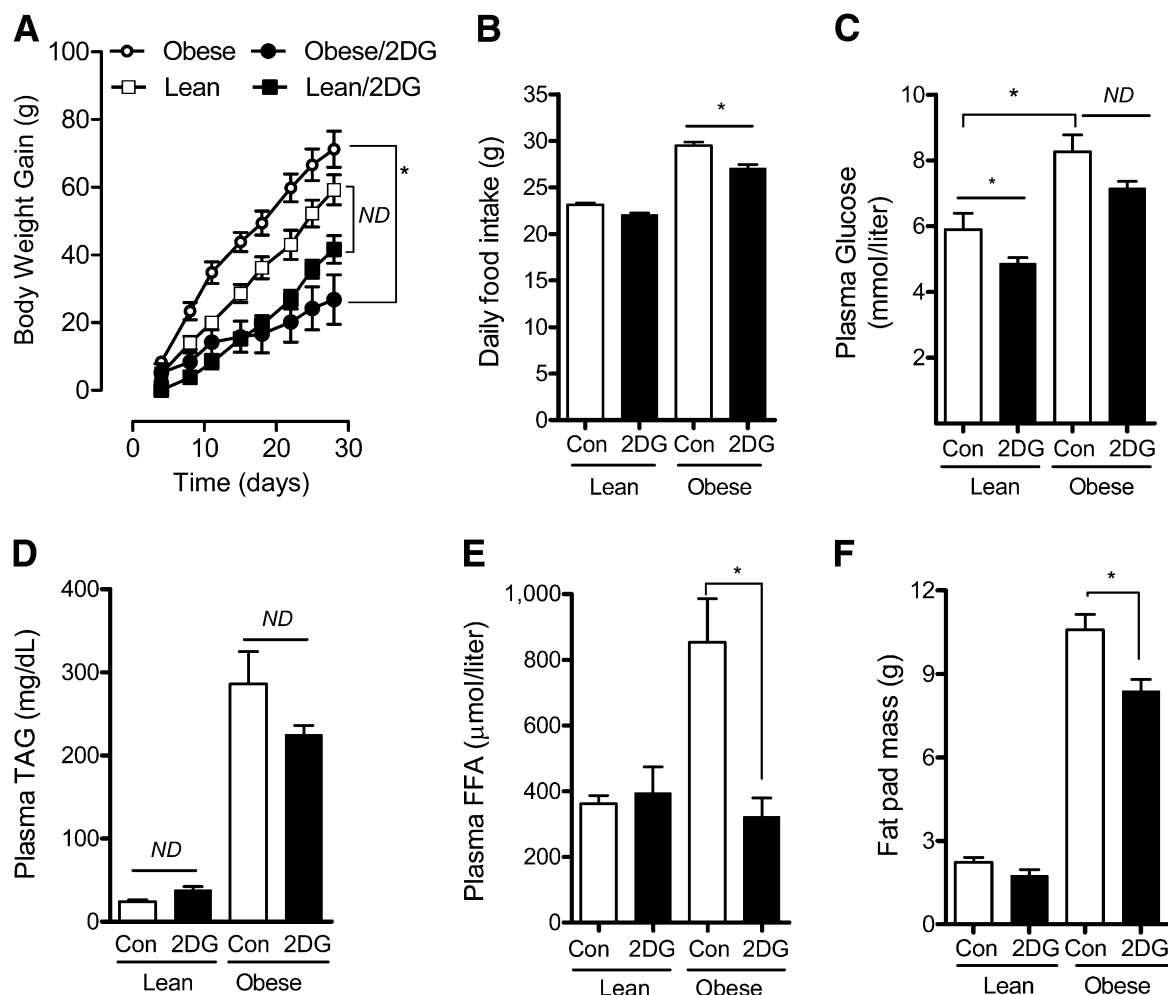


FIG. 6. Obese *fa/fa* Zucker rats maintained on a 2DG-supplemented diet gain less body weight and exhibit reduced plasma FFAs. Ten-week-old male lean *Fa/fa* and obese *fa/fa* Zucker rats were maintained on an ad libitum standard chow diet (Con) or one supplemented with (0.4% w/w) 2DG for 28 days. Corresponding changes in body weight gain (A) and mean daily food intake (B) for each animal genotype/diet group were monitored twice weekly and daily, respectively. Plasma glucose (C), triacylglycerol (TAG) (D), and total FFAs (E) were measured using blood specimens from anesthetized rats after an overnight fast as described in RESEARCH DESIGN AND METHODS. Average epididymal fat pad weights from each animal genotype/diet group are presented (F). All data are expressed as mean \pm SEM ($n = 5$ /group). * $P < 0.05$, obese 2DG vs. obese control. ND, no difference.

reduced insulin sensitivity (reviewed in 3,4). Indeed, studies in rodents and cultured myotubes have demonstrated that inhibiting enzymes involved in endogenous ceramide synthesis (e.g., dihydroceramide desaturase-1 and serine palmitoyl transferase) not only improves glucose homeostasis but also restrains the insulin-desensitizing effects of PA (6,31). Intriguingly, while suppressing de novo ceramide synthesis from PA is insulin sensitizing in the short term, sustained loss of serine palmitoyl transferase activity in myotubes induces greater partitioning of PA toward DAG biosynthesis, which then promotes insulin resistance via PKC-mediated serine phosphorylation of IRS-1 (6). These observations support a significant role for DAG and ceramides in fatty acid-induced insulin resistance. However, the current findings demonstrate that muscle cells held in glucose-free media are protected against the deleterious effects of PA despite increases in intramyocellular DAG and ceramide. These findings imply that the lipotoxic effect of these lipids can be muted under certain circumstances and add to the current debate as to whether their accumulation is inextricably linked to development of tissue insulin resistance (32–34). Our studies indicate that glucose or its metabolites may play a permissive role in the

pathogenic action of DAG and ceramide. Glucose availability/metabolism may not only influence accumulation of select DAG/ceramide species but also result in these lipids or their signaling effectors being spatially segregated from molecules mediating insulin and proinflammatory signaling. The finding that cell-permeable ceramide impairs Akt activation, irrespective of whether myotubes are held in media containing glucose, supports the “segregation” hypothesis. Moreover, we have previously demonstrated that a critical feature underpinning inhibition of Akt in response to ceramide is recruitment and retention of the kinase within caveolin-enriched microdomains (CEMs) (35). This targeting to CEMs is, in turn, dependent upon caveolar recruitment of atypical PKCs, which are potently activated by ceramide and which physically interact with Akt and caveolin. Intriguingly, we find that while PA induces recruitment of atypical PKCs to CEMs in muscle cells, this recruitment is substantially reduced in PA-treated muscle cells that have been coincubated with 2DG (Supplementary Fig. 4). It is currently unclear how 2DG restrains PA-induced caveolar targeting of PKCs, but the observation implies that the inability to relocalize atypical PKCs to CEMs under glucopenic conditions may,

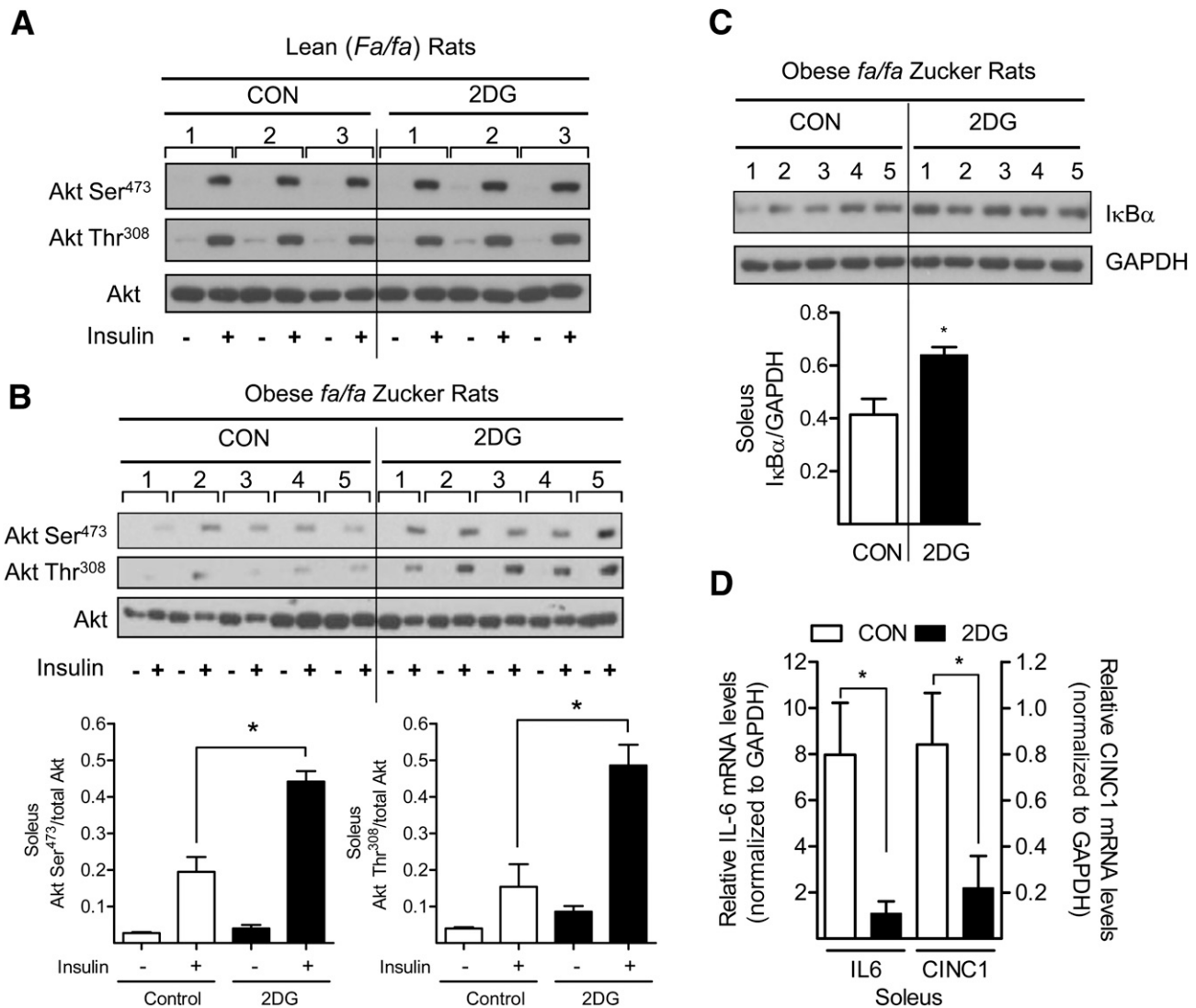


FIG. 7. Obese Zucker rats maintained on a 2DG-supplemented diet exhibit improved skeletal muscle insulin sensitivity and reduced cytokine expression. Ten-week-old lean (*Fa/fa*) and obese *fa/fa* Zucker rats were maintained on an ad libitum standard chow diet (CON) or one supplemented with 2DG (0.4% w/w) for 28 days. After overnight starvation, rats were anesthetized immediately prior to receiving an intravenous injection of insulin (10 units/kg) for 5 min. Solei were isolated from each animal as described in RESEARCH DESIGN AND METHODS. Insulin signaling (total Akt, phosphorylated Ser⁴⁷³ Akt, and phosphorylated Thr³⁰⁸ Akt) was measured using lysates prepared from solei isolated 5 min after insulin injection. (A: Lean animals, $n = 3$ /group. B: Obese animals, $n = 5$ /group.) For each animal, the phosphorylated Akt signal in each lane was normalized to total Akt in that lane. These normalized values were then averaged from the 5 animals in each group to compare insulin-induced phosphorylation of Akt (Ser⁴⁷³ and Thr³⁰⁸) in muscle of obese animals on the control diet versus that of obese animals on the 2DG diet (B, lower panel). C: Lysates from non-insulin-stimulated solei from obese animals were used for immunoblot analysis of proinflammatory signaling (IκBα, glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) (B) ($n = 5$ /group). Total RNA extracted from non-insulin-stimulated solei was used to determine IL-6 and CINC-1 (D) relative to GAPDH mRNA expression by qPCR analysis (C) ($n = 5$ /group). All values are presented as mean \pm SEM. * $P < 0.05$, obese 2DG vs. obese control (corresponding insulin treatment in B) or between indicated bar values.

in part, explain how the inhibitory effects of PA on Akt signaling are attenuated despite a prevailing intracytoplasmic increase in PA-derived ceramide.

It is plausible that the protective effect conferred by glucose withdrawal/2DG supplementation against the proinflammatory potential of PA may be linked to suppressing effects that the fatty acid may have upon either reactive oxygen species formation or signaling initiated via Toll-like receptor 4 (TLR4). However, we are able to discount the notion that reactive oxygen species may be involved given that application of antioxidants (N-acetylcysteine and vitamin C) did not curtail PA-induced activation of the ERK-IKK signaling axis (Supplementary Fig. 5). The involvement of TLR4 in our cells can also be excluded, given that while LPS (a TLR4 ligand) induced IKK-directed signaling in bone marrow

dendritic cells, it had no observable effect on proinflammatory NF-κB signaling in our muscle cells (Supplementary Fig. 6).

Mitochondrial dysfunction associated with FFA overload has been implicated in the pathogenesis of skeletal muscle insulin resistance (36,37). Koves et al. (38) demonstrated that increasing FFA oxidation by chronic high-fat feeding or genetic obesity in mice imposes a major substrate load on muscle's oxidative machinery that eventually surpasses the respiratory drive for ATP synthesis. Under these circumstances, FFAs undergo incomplete oxidation resulting in accumulation of acylcarnitines, which then promote insulin resistance by mechanisms that currently remain unclear (38). Accumulation of acylcarnitines is, nonetheless, likely to be a secondary consequence of reduced mitochondrial respiratory drive brought about by

the impact that sustained fatty acid overload has upon production of reactive oxygen species (39,40) and genes encoding PGC-1 α and respiratory chain components that, together, will reduce the abundance, function, and integrity of mitochondria (12,13,16). Consistent with previous studies, we observe a significant decline in PGC-1 α expression and mitochondrial respiration but, importantly, demonstrate that these are a likely consequence of being unable to deal with not only the carbon load born from PA but also that originating from glucose. Moreover, the improved insulin sensitivity and reduced proinflammatory drive conferred by glucose withdrawal/glycolytic inhibition requires PA oxidation, since the metabolic benefits associated with glucose restriction are antagonized by inhibiting mitochondrial PA uptake. Equally, it is noteworthy that inhibiting fatty acid entry (and, hence, influx of fatty acid-derived carbon) into mitochondria when glucose is available averts the insulin-desensitizing effect associated with fatty acid overprovision (38). Together, these observations imply that, irrespective of which metabolic fuel is available, as long as the total or combined fuel load does not exceed mitochondrial oxidative capacity and respiratory drive is maintained within a normal threshold, the fidelity with which insulin activates molecules such as Akt is likely to be upheld.

Our work demonstrates that the ability to sustain PA oxidation and preserve insulin sensitivity when glucose availability is restricted depends on maintaining mitochondrial anaplerosis. Inhibiting transaminases and pyruvate carboxylase, which support anaplerotic generation of OAA, would ultimately “run down” the TCA cycle with a concomitant decline in respiratory activity and energy balance. The failure to oxidize PA would then reinstate the insulin-desensitizing and proinflammatory potential of PA. Since inhibiting mitochondrial anaplerosis will promote incomplete fatty acid oxidation, we cannot exclude the possibility that impaired insulin signaling seen under these conditions is attributable to intramitochondrial acylcarnitine accumulation (38).

Reduced calorie intake has been shown to increase PGC-1 α expression, mitochondrial density, oxygen consumption, and ATP production (41,42). These beneficial gains have been linked to sirtuin 1 (SIRT1), which deacetylates PGC-1 α , thereby enhancing transcription of mitochondrial oxidative genes (43). AMP kinase (AMPK) has been implicated as a SIRT1 activator (44), and very recent work indicates that mice lacking AMPK α 2 in skeletal muscle lose the insulin-sensitizing effect associated with calorie restriction (45). Since glucose withdrawal and 2DG supplementation are known to stimulate AMPK and the activated kinase supports mitochondrial fatty acid uptake by reducing cytosolic levels of malonyl CoA (via phosphorylation/inhibition of acetyl CoA carboxylase), it is plausible that the insulin-sensitizing and anti-inflammatory effect associated with glucose withdrawal may involve AMPK. In line with this possibility, we find that muscle cells in which AMPK expression/activity has been stably silenced by ~90% (46) exhibit a compromised response to glucose withdrawal in terms of repressing the PA effect upon Akt activation and I κ B α loss (Supplementary Fig. 7). It is conceivable that because myotubes still express some residual, albeit small (~10%), measure of AMPK activity or SIRT1 is activated via AMPK-independent mechanisms that this may account for some of the salutary effects of glucose withdrawal that remain in the AMPK-silenced myotubes. However, while activation of SIRT1 by such mechanisms cannot be excluded, two different deacetylase inhibitors

failed to suppress the protective effect conferred by glucose withdrawal upon Akt and I κ B α when myotubes were overloaded with PA (Supplementary Fig. 8).

Since calorie restriction preserves mitochondrial function and tissue sensitivity to insulin, the development of calorie restriction mimetics may offer an effective approach for combating insulin resistance and tissue inflammation in conditions such as diabetes and obesity. In accord with our cell-based observations and carbohydrate-restriction studies (47), we demonstrate that dietary supplementation of 2DG as a calorie restriction mimetic (48) in an obese rodent model induces a dramatic reduction in circulating FFAs that is associated with improved insulin-dependent Akt phosphorylation and reduced inflammatory tone in skeletal muscle. While we cannot exclude the possibility that the reduction in plasma FFAs may be a consequence of reduced lipolysis, the finding that epididymal fat pad mass was reduced significantly in 2DG-fed obese animals implies that the fall in circulating FFAs may be linked to their greater oxidation in tissues such as muscle. Whether these 2DG-driven responses *in vivo* are a direct consequence of enhanced mitochondrial function via, for example, the AMPK–PGC-1 α axis is currently unclear, but testing this possibility more thoroughly is a major goal of future work.

In summary, our report reveals that chronic overprovision of PA induces a marked reduction in insulin action and heightened proinflammatory signaling in skeletal muscle. These responses are associated with mitochondrial dysfunction that most likely stems from an inability to match oxidative capacity to excess fuel supply. Importantly, reducing the carbon load on mitochondria by glucose withdrawal or glycolytic inhibition helps preserve the ability to oxidize PA and restrains its insulin-desensitizing and proinflammatory potential in muscle cells. In support of this concept, a glucopenic diet proffered to obese insulin-resistant animals induces beneficial gains in skeletal muscle insulin action and inflammatory status. Together, these observations indicate that strategies that maintain/improve mitochondrial oxidative capacity may help limit metabolic dysfunction associated with fatty acid overload in skeletal muscle.

ACKNOWLEDGMENTS

This work was supported by grants from the Biotechnology and Biological Sciences Research Council and Diabetes UK to H.S.H.; and, in part, by funds from the German Research Foundation to C.W. (GRK 1302/2) and from the German Federal Ministry of Education and Research (BMBF) to the German Centre for Diabetes Research (DZD e.V.).

No potential conflicts of interest relevant to this article were reported.

C.L., K.M., and T.S. participated in the design, execution, and analysis of experimental work and data. C.W. was involved in conceiving the experimental/research program, data interpretation, and manuscript preparation and editing. A.B.-Z. and M.B. participated in the design, execution, and analysis of experimental work and data. J.G. was involved in conceiving the experimental/research program, data interpretation, and manuscript preparation and editing. K.B. participated in the design, execution, and analysis of experimental work and data. H.S.H. was involved in conceiving the experimental/research program, data interpretation, and manuscript preparation and editing. H.S.H. is the guarantor of this work and, as such, had full access to

all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Heike Runge (University of Tübingen) for excellent technical assistance and Dr. Clare Stretton (University of Dundee) for useful discussions.

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