Downregulation of AMPK accompanies leucine- and glucose-induced increases in protein synthesis and insulin resistance in rat skeletal muscle

Asish K. Saha1*, X. Julia Xu1, Ebony Lawson1, Rosangela Deoliveira1, Amanda E. Brandon2, Edward W. Kraegen2 and Neil B. Ruderman1

1Endocrinology and Diabetes, Department of Medicine, Boston University Medical Center, Boston, Massachusetts; 2Diabetes and Obesity Program, Garvan Institute of Medical Research and School of Medical Sciences, University of NSW, Sydney, Australia.

*To whom correspondence should be addressed at:
Asish K. Saha
Diabetes and Metabolism Unit
E-mail: aksaha@bu.edu

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Objectives—Branched-chain amino acids (BCAA), such as leucine, and glucose stimulate protein synthesis and increase the phosphorylation and activity of the mammalian target of rapamycin (mTOR) and its downstream target p70S6 kinase. We examined in skeletal muscle whether the effects of leucine and glucose on these parameters and on insulin resistance are mediated by the fuel-sensing enzyme AMP-activated protein kinase (AMPK).

Research design and methods—Rat extensor digitorum longus (EDL) muscle incubated with different concentrations of leucine and glucose ± AMPK activators and muscle obtained from glucose infused rats were used as models.

Results—In the EDL, incubation with 100 or 200 μM of leucine versus no added leucine suppressed the activity of the α2 isoform of AMPK by 50 and 70% respectively and caused concentration-dependent increases in protein synthesis and mTOR and p70S6 kinase phosphorylation. Very similar changes were observed in EDL incubated with 5.5 or 25 mM vs. no added glucose and in muscle of rats infused with glucose in vivo. Incubation of the EDL with higher concentrations of both leucine and glucose also caused insulin resistance as reflected by a decrease in insulin stimulated Akt phosphorylation. Co-incubation with the AMPK activators AICAR and α-lipoic acid (ALA) substantially prevented all of those changes and increased the phosphorylation of specific sites of mTOR inhibitors, raptor and TSC2. In contrast, decreases in AMPK activity induced by leucine and glucose were not associated with a decrease in raptor and TSC2 phosphorylation.

Conclusions—The results indicate that both leucine and glucose modulate protein synthesis and mTOR/p70S6 and insulin signaling in skeletal muscle by a common mechanism. They also suggest that the effects of both molecules are associated with a decrease in AMPK activity and that AMPK activation prevents them.

AMPK is a fuel sensing enzyme that has classically been defined in terms of its role in restoring ATP levels in energy depleted cells. In skeletal muscle, AMPK is typically activated by such factors such as glucose deprivation and contraction (exercise) (1, 2). The activated AMPK in turn enhances processes that generate ATP, such as fatty acid oxidation and glucose transport, and downregulates other processes that consume ATP and can be diminished temporarily without jeopardizing the cell (eg protein and lipid synthesis). Much less studied is the notion that a decrease in AMPK below baseline values may also be a physiologically or pathophysiologically relevant event. In keeping with such a possibility decreased AMPK activity has been observed in tissues of many obese insulin-resistant rodents (3) and in liver (4, 5) and adipose tissue (6) of 48h starved rats when they are refed. One consequence of decreased AMPK activity could be increases in mTOR/p70S6K signaling and protein synthesis since both are decreased by AMPK activation (7).

In the present study, we assessed whether fuel-induced increases in protein synthesis, mTOR/p70S6K signaling and insulin resistance in skeletal muscle are mediated by decreases in AMPK activity. Toward this end, rat EDL muscles were incubated for different time periods with various concentrations of leucine or glucose and the above parameters were assessed. The
results indicate that elevated concentrations of leucine and glucose decrease AMPK activity, increase protein synthesis and mTOR/ p70S6 phosphorylation and cause insulin resistance and that activation of AMPK by pharmacological agents prevents these events from occurring. Finally, the data suggest that the decrease in AMPK activity caused by both leucine and glucose is not mediated by changes in the AMP/ATP ratio but is associated with an increase in the lactate/pyruvate ratio.

MATERIALS AND METHODS
Chemicals and materials. AICAR was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). Compound C, a selective AMPK inhibitor, from Calbiochem, San Diego, CA. [γ-32P] ATP from NEN, Boston, MA and Protein A/G plus conjugate from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for P-AMPK (Thr 172), total AMPK, P-mTOR (Ser 2448), P-p70S6K (Thr 389) and P-4EBP (Thr 70) were obtained from Cell Signaling (Danvers, MA) and P–ACC (Ser 79) from Upstate Biotechnologies (Charlottesville, VA). P-TSC2 (Ser1387) from LifeSpan Biosciences, Inc, Seattle, WA. Total and P-raptor (Ser 792), total TSC2 and Rabbit polyclonal anti-SIRT1 (H-300) were from Santa Cruz Biotechnology, Santa Cruz, CA. “SAMS” peptide and polyclonal antibodies that immunoprecipitrate the α1 and α2 catalytic subunit of AMPK were obtained from QCB biotechnology (Hopkinton, MA). All other chemicals were purchased from either Sigma-Aldrich or Fisher.

Experimental animals. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of Boston University Medical Center and were in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats weighing 55–65 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). They were maintained on a 12:12-h light-dark cycle in a temperature-controlled (19-21°C) room and were fed standard Purina rat chow and water ad libitum. Food was withdrawn 16-20 h before the initiation of experimental protocols. Muscles were removed from rats anesthetized with pentobarbital (6mg/100g bw).

Muscle incubation. After removal from the rat extensor digitorum longus (EDL) muscles were first equilibrated for 20 min at 37°C in oxygenated Krebs-Henseleit solution (95% O2/5% CO2) containing 5.5 mM glucose (8). They were then incubated in media containing 0, 5.5, or 25 mM glucose and different concentrations of leucine (0-200 μM). for varying time periods as indicated in the Figure legends. (Physiological concentration of leucine is usually 70-120 μM) (9, 10). At the end of the incubation, muscles were blotted, quick-frozen in liquid nitrogen and stored at -80°C until used for analyses.

AMPK activity assay. AMPK activity was measured in the EDL as described previously (8, 11). In brief, frozen muscle was homogenized (20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM Na4P2O7, 100 mM NaF, 2 mM Na3VO4, 1% NP-40, 1 mM PMSF, 0.006 mg/ml Aprotinin, 0.006 mg/ml Leupeptin) and the muscle lysate containing 200-μg protein was immunoprecipitated with specific antibodies to the α-2 or α-1 catalytic subunit of AMPK (11) and protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed five times, and the immobilized enzyme was assayed based on the phosphorylation of SAMS peptide (0.2 mmol/l) by 0.2 mmol/l ATP (containing 2 μCi [γ-32P] ATP) in the presence and absence of 0.2 mmol/l AMP. Label incorporation into the SAMS peptide was measured on a Racbeta 1214 scintillation counter.

Western blot analysis. Protein homogenates (50 μg) were run on a 4-15% gradient SDS polyacrylamide gel (BioRad) and transferred onto a PVDF (polyvinylidene fluoride)
membrane (Bio-Rad). Membranes were then stained with Ponceau S (1% in 5% acetic acid) to ensure even transfer and blocked in Tris-buffered saline (pH 7.5) containing 0.05% Tween-20 (TBST) and 5% milk for 1 h at room temperature. After this they were incubated overnight in primary antibodies (P-AMPK, P-ACC, total AMPK, P-mTOR, P-p70S6K, P-raptor and P-TSC2) at a 1:1,000 dilution. They were then incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham) at a 1:5,000 dilution and subjected to an enhanced chemiluminescence solution (Pierce). Densitometry was performed using Scion Image software (8).

**Assessment of protein synthesis.** EDL were initially equilibrated in Krebs-Henseleit solution containing 5.5 mmol/l glucose for 20 min. They were then incubated for 1 h in fresh medium containing 2 mCi/ml of 14C-phenylalanine, unlabelled phenylalanine at a final concentration of 100 μM/ml and the indicated concentrations of glucose and leucine. At the end of the incubation, muscles were blotted and homogenized in 10% TCA. Samples were centrifuged at 10,000Xg for 10 min at 4°C and TCA-insoluble material was washed 3 times with 10% TCA. The resultant pellet was solubilized in 0.1N NaOH at 37°C for 2 hours and used for determination of protein abundance and phenylalanine incorporated into muscle protein (7). Protein mass was determined by the BCA procedure (see below) and protein-associated radioactivity by liquid scintillation counting. Protein synthesis was calculated by dividing the protein-bound radioactivity by the specific activity of free leucine in the incubation medium. The results are expressed as nmol of leucine incorporated per milligram protein per hour.

**Glucose Infusion.** Glucose infusion was carried out as described previously (12). Briefly, seven days after cannulation surgery, rats were randomly divided into treatment groups. After a basal blood sample (600μl) was taken and a 50% (w/v) glucose solution was infused for either 0 or 5h using a peristaltic roller pump (101U/R; Watson-Marlow, Falmouth, UK). Blood samples were taken every 30 min and the glucose infusion rate was altered to maintain a blood glucose concentration of 11mM (~16-17mM plasma glucose).

**Other analyses.** Protein concentrations were determined with the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard. ATP, AMP, ADP, and phosphocreatine were measured spectrophotometrically as described previously (8). Malonyl CoA was determined radioenzymatically by a slight modification of the method of McGarry et al. (13). Lactate and pyruvate was determined spectrophotometrically using lactate dehydrogenase and NAD (8, 14).

**Statistics.** Results are expressed as means ± SEM. Statistical differences between two groups were determined by the Student’s t-test where multiple groups were compared by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc analysis. Differences between groups were considered statistically significant at P< 0.05.

**RESULTS**

**Incubation with leucine increases protein synthesis and the phosphorylation of mTOR, p70S6K and 4EBP1.** In keeping with previous reports in muscle (15) and pancreatic β cells (16), incubation of the EDL with leucine (100 and 200 μM) for 1 h significantly increased protein synthesis (Figure 1A), an effect associated temporally with increases in the phosphorylation of mTOR and p70S6 kinase, but not another mTOR target, 4-EBP1 (Figure 1B and C). In the presence of 100 μM isoleucine we found 20% increases in both mTOR and p70S6K phosphorylation; however, neither achieved statistical significance (Figure S1 in the online
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Leucine concurrently diminishes AMPK phosphorylation and activity. EDL incubated with leucine for 1 h also demonstrated a significant decrease in the phosphorylation of AMPK at Thr172. As shown in Figure 2A, a progressive decrease in the abundance of both P-AMPK and its downstream target ACC occurred as the concentration of leucine in the medium was increased from 0 to 50, 100 and 200 μM. An almost identical pattern was observed when the activity of the α2 isoform of AMPK was measured (Figure 2B). In contrast, the activity of α1 AMPK was unchanged (data not shown). Time-course studies revealed that 100 μM leucine decreased AMPK phosphorylation by 20% at 15 min, 40% by 30 min and 80% after 2 hour (Figure 2C). Incubation with 100 μM isoleucine another branched chain amino acid, also decreased AMPK activity, although not to the same extent as leucine. In contrast, incubation with 100 μM glutamine had no effect (Figure 2D).

Incubation with glucose mimics the effects of leucine on protein synthesis, mTOR/p70S6k phosphorylation and AMPK phosphorylation and activity. Incubation with glucose had effects very similar to those of leucine. As shown in Figure 3A, protein synthesis increased by more than 2-fold when the medium concentration of glucose was increased from 5.5 to 25 mM as did the phosphorylation of mTOR and p70S6K. Conversely, eliminating glucose from the medium decreased all of these parameters (Figure 3B). As with leucine, no change in the phosphorylation of 4-EBP1 was observed. Incubation of the EDL with higher concentrations of glucose (25 vs 5.5 vs 0 mM) for 1hr also decreased the phosphorylation of AMPK and ACC (Figure 4) and the activity of the α2 isoform of AMPK (data not shown). In contrast, eliminating glucose from the medium increased all of these parameters. No change in the activity of α1 AMPK was observed under these conditions (data not shown). The concentration of malonyl CoA changed inversely with the activity and phosphorylation of AMPK as described previously (11).

AICAR and another AMPK activator α-lipoic acid (ALA) inhibit glucose induced changes in protein synthesis and AMPK and mTOR/p70S6 kinase phosphorylation. The above mentioned results indicate that the ability of both leucine and glucose to increase mTOR signaling and protein synthesis is associated with a decrease in AMPK phosphorylation and α2 AMPK activity. To assess whether AMPK downregulation might be the cause of these changes, studies were performed in which AMPK downregulation was prevented by co-incubation with the AMPK activators, AICAR or ALA. In muscles incubated with 5.5 mM glucose for 1 hr, AICAR (1 mM) decreased protein synthesis by 50% to a rate similar to that observed in muscles incubated in a glucose-free medium (Figure 5A). As shown in Figure 5B, AICAR activated α2-AMPK, but not α1-AMPK and it increased the phosphorylation of AMPK and ACC and decreased the concentration of malonyl CoA (Figure 5C). In addition it diminished the phosphorylation of both mTOR and p70S6K (Figure 5D). Almost identical findings were observed when the EDL was incubated with α-lipoic acid (ALA), a naturally occurring short-chain fatty acid, that has been shown to activate AMPK in muscle by increasing CAMKKβ (17). Thus, at 5 mM glucose, incubation with 100 μM ALA increased the phosphorylation of AMPK by 3-fold (see the Online-Appendix, Figure S2A) and decreased both mTOR phosphorylation (Online-Appendix, Figure S2B) and protein synthesis by 50% (Online-Appendix, Figure S2C). As shown below in Figure 7, AICAR prevented the inhibition of insulin-stimulated PKB/Akt
phosphorylation caused by high glucose and leucine. In order to determine whether this effect is not specific to AICAR, we treated EDL muscle with ALA in presence of high glucose. As shown in the Online-Appendix, Figure S2D, the decrease in Akt phosphorylation by high glucose was completely prevented by ALA.

**Effects of AICAR on mTOR phosphorylation and protein synthesis in EDL incubated with 25 mM glucose or leucine**

As shown in Figure 6A, AICAR prevented, although not completely, the decreases in AMPK phosphorylation and the increases in mTOR phosphorylation (Figure 6B) and protein synthesis (Figure 6C) caused by incubating muscle with 25 vs 5 mM glucose. It also prevented the changes in these parameters caused by incubation with leucine (Figure 6D).

**AMPK activation also diminishes leucine and glucose induced insulin resistance.**

Incubation with leucine has also been shown to cause insulin resistance in skeletal muscle (15). In keeping with this observation we found that the ability of insulin to increase the phosphorylation of Akt on Ser 473 was diminished by 50% in EDL incubated with leucine at a concentration of 100 μM. As shown in Figure 7, this effect of leucine was completely prevented by co-incubation with 1 mM AICAR. An identical observation was observed when EDL was incubated with 25 mM glucose and AICAR prevented the decrease in AMPK phosphorylation caused by high glucose (data not shown).

**Effect of Compound C on AMPK and mTOR phosphorylation at 5 mM glucose.**

The studies with AICAR and ALA suggest that AMPK activation prevents or partially prevents both the decrease in AMPK activity and the increase in mTOR/p70S6 kinase phosphorylation and protein synthesis caused by leucine and glucose. To assess further whether decreased AMPK plays a causal role in mediating these changes, we next evaluated whether the effects of glucose and leucine on these parameters could be mimicked by incubating the EDL with 50 μM of Compound C, a selective AMPK inhibitor. As shown in the Online-Appendix, Table S1, Compound C did not affect P-AMPK or mTOR phosphorylation and protein synthesis under baseline conditions indicating it could not be used for this purpose.

**Examination of factors that could mediate the downregulation of AMPK by leucine and high glucose**

(a) **Energy state and lactate and pyruvate concentration**

In a search for factors responsible for the decrease in AMPK phosphorylation caused by leucine and glucose, we first assessed cellular energy state. Incubation with 100 μM leucine for 30 min (not shown) or 1 h had no effect on whole tissue levels of creatine phosphate, ATP, ADP or AMP (Table 1); however it increased muscle lactate and to a lesser extent pyruvate. The net result was a nearly 2-fold increase in the lactate/pyruvate ratio (Table 2). In keeping with our previous report (8), no differences in the whole-tissue concentrations of creatine phosphate, ATP, ADP or AMP were observed in muscles incubated with 25 vs 5 mM glucose for 1 h and here too tissue lactate and pyruvate were increased as was the lactate/pyruvate ratio (Table 2).

The observed changes in the lactate/pyruvate ratio suggests that leucine and high glucose both cause increases in NADH relative to NAD⁺ in muscle. For this reason we examined the effects of incubation with glucose or leucine on the abundance of the NAD⁺ dependent, redox-sensitive histone protein deacetylase SIRT1. In a previous study in HepG2 cells (18), we found that incubation with 25 vs 5 mM glucose caused similar changes in cell lactate and significantly diminished SIRT1 abundance (by 20%). In the present study, SIRT1 protein was also decreased by 20% in the muscle incubated with 25 mM vs 5.5 glucose;
however, the decrease was not statistically significant (p<0.12) (online-Appendix S 4).

(b) Downregulation of mTOR/p70S6 kinase signaling by rapamycin We next assessed whether the decrease in AMPK phosphorylation caused by leucine was dependent on its ability to activate mTOR. Toward this end, EDL were incubated with 200μM of leucine in the presence or absence of rapamycin, an inhibitor of mTOR signaling. As shown in Online-Appendix, Figure S3, rapamycin inhibited the increased phosphorylation of mTOR and p70S6 kinase induced by leucine, but it did not prevent the decrease of AMPK phosphorylation caused by leucine (Online-Appendix, Figure S2).

(c) Phosphorylation of raptor and TSC2 in EDL muscle treated with AICAR, high glucose and leucine Recently, AICAR-induced activation in myocytes and other cells has been reported to impair TORC1 kinase activity by increasing the phosphorylation of raptor on Ser792 (19). As shown in Online-Appendix, Figure S5A, Ser792 phosphorylation of raptor in EDL incubated with 1 mM AICAR was 3-fold higher than in the control group. In contrast, high glucose and leucine at concentrations that diminished AMPK phosphorylation and activity did not decrease raptor phosphorylation, nor did they diminish the increase in raptor phosphorylation caused by AICAR (Online-Appendix, Figure S5B).

A second mechanism by which AMPK might diminish TORC1 signaling and protein synthesis is by phosphorylating tuberous sclerosis complex 2 (TSC 2) on Ser 1387 (20). In the incubated EDL, AICAR increased TSC2 phosphorylation by 4-fold under basal conditions and 2-3 fold in the presence of high glucose and leucine. However, neither high glucose nor leucine by themselves, diminished TSC2 phosphorylation from control values (Online-Appendix, Figure S6).

Effects of a glucose infusion in vivo. To determine whether the effects of glucose observed in the incubated EDL also occur in vivo, rats were infused with 50% glucose for 5h with the rate of infusion adjusted to maintain the plasma glucose concentration at 16-17 mM. The plasma insulin concentration was 250 μU/L during the infusion vs 50 μU/L prior to its start. As shown in Figure 8, AMPK α2 activity in red gastrocnemius muscle was decreased after 5h of glucose infusion compared to baseline values and the phosphorylation of mTOR and p70S6 kinase was increased. It has previously been demonstrated that the glucose-infusion also caused insulin resistance by 5 h (12).

DISCUSSION
The physiological and biochemical relevance of AMPK activation has been well described (21-24). In contrast, less is known about the consequences of AMPK downregulation. In the present study, we show that excesses of glucose and the branched chain amino acid leucine stimulated protein synthesis and cause insulin resistance in skeletal muscle and that both effects were paralleled by decreases in AMPK activity. Furthermore, activation of AMPK with both AICAR and α-lipoic acid (ALA) prevented these events from occurring.

That leucine at physiological concentrations (70-120 μM) stimulates protein synthesis (25, 26) and causes insulin resistance (27) in skeletal muscle and that it does so by increasing mTOR/p70S6K signaling (28) has been reported previously. The novel findings of the present study are that these changes are associated with a decrease in AMPK activity and that they are prevented by incubation with two AMPK activators that work by different mechanisms. Similar observations have recently been made in the pancreatic β-cell (16) suggesting that a decrease in AMPK activity might also contribute to the well documented stimulation of insulin synthesis and secretion by branched-chain amino acids. The results also
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indicate that these effects are not unique to leucine since incubation of the EDL with progressively higher concentrations of glucose produced an almost identical pattern of events. Collectively these findings suggest the existence of a common fuel sensing and signaling mechanism by which leucine and glucose downregulate AMPK and alter protein synthesis, insulin sensitivity and presumably other processes as their concentration is increased (Online-Appendix, Figure S7).

The question of whether the decreased AMPK activity induced by leucine and glucose mediated their effects on mTOR/p70S6K signaling and secondarily protein synthesis is unresolved. On the one hand, increased AMPK activity has been shown to inhibit protein synthesis both by phosphorylating and activating tuberous sclerosis complex 2 (TSC2) and raptor (19, 20), findings that we confirmed in the incubated EDL. Likewise, the decrease in AMPK activity correlated very strongly with both the increases in protein synthesis and mTOR/p70S6K signaling. On the other hand, if decreased AMPK modulated these effects, one would predict that phosphorylation of the AMPK sensitive sites on TSC2 and raptor would be diminished. This was not found. Incubation with high glucose and leucine both failed to decrease the phosphorylation of raptor on Ser792 and TSC2 on Ser1387. Since baseline phosphorylation of these molecules was very low the possibility that a significant decrease was missed cannot be excluded. Likewise, it is possible that the decrease in AMPK caused by leucine and glucose altered other molecules that could upregulate mTOR/p70S6K (reviewed by 20). Clearly the role of decreased AMPK in mediating the increase in protein synthesis caused by these molecules will require further study.

The findings also raise the question of by what mechanism does an excess of two different fuels lead to a decrease in AMPK activity? The decreases in AMPK activity induced by both leucine and glucose were not associated with an increase in energy state; indeed, the whole tissue concentrations of creatine-P and the AMP/ATP ratio were the same at all glucose and leucine concentrations tested. That a local decrease in the AMP/ATP ratio occurred that was not reflected in the whole tissue cannot be ruled out, however. Perhaps relevant to this discussion, we found 2-3 fold increases in the lactate/pyruvate ratio in muscle incubated with both high glucose and leucine (Table 2), indicating increases in the cytosolic and presumably the nuclear redox state (i.e. a decreased NAD+/NADH ratio). This in turn was associated with a 20% decrease in the abundance of the NAD+-dependent histone/protein deacetylase SIRT1, an enzyme reported to deacetylate the AMPK kinase LKB1 leading to an increase in its activity and that of AMPK (29-31). On the other hand, the decrease in SIRT1 abundance observed here was not statistically significant (p<0.12, n=6). This contrasts with an earlier study, in which we incubated HepG2 cells in a high glucose medium (25 vs 5 mM), and observed similar decrease in AMPK phosphorylation and SIRT1 abundance (20%) both of which were statistically significant (18). Clearly further investigations are needed to assess whether glucose and leucine affect SIRT1 in the EDL. Finally, incubation of the EDL with the mTOR inhibitor, rapamycin, had no effect on the ability of leucine to diminish AMPK phosphorylation (Online-Appendix, Figure S3). Thus, the decrease in AMPK activity was not a consequence of mTOR activation.

With respect to the relevance of the findings to muscle in vivo, the infusion of glucose at a rate sufficient to increase its plasma concentration to 16-17 mM and plasma insulin levels from 50 to 250 mU/ml increased both mTOR/p70S6k signaling and decreased AMPK activity after 5 h (Figure 8). Others have shown that feeding a high protein
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diet (ingestion of branched chain amino acids) causes similar changes in AMPK and mTOR/p70S6K in liver (32). Presumably, these events occur in muscle and liver physiologically after meals dependent on their composition. Whether the glucose and leucine-induced decreases in AMPK activity contribute to the post-prandial increases in protein (mTOR/p70S6K) synthesis is open to debate as it is in the incubated EDL. On the other hand, it is highly likely that concurrent changes in lipid synthesis in muscle during a glucose infusion (12) are due to the decrease in AMPK since they are associated with decreased phosphorylation and increased activity of the AMPK target, acetyl CoA carboxylase (ACC). Also, in keeping with this conclusion, in the present study we found that the concentration of the ACC product malonyl CoA was elevated in the EDL when it was incubated with leucine or high glucose (see Figs 4 and 5).

As already noted, incubation of muscles with elevated concentrations of leucine (27) or glucose (8) have been shown to cause insulin resistance. The results presented here suggest that the insulin resistance correlates with a decrease in AMPK activity; however, for the same reason discussed in the context of mTOR/p70S6K signaling and protein synthesis, it is unclear whether the decrease in AMPK was a causal event. What ever the mechanism we would propose that such fuel-induced insulin resistance in vivo develops post-prandially when the muscle or liver cell senses its need to synthesize glycogen or protein has been met and that it is to the benefit of the organism to shunt glucose and amino acids to other tissues (eg adipose tissue) for storage. By virtue of the setting in which it occurs, and its likely reversibility, one might refer to this phenomenon as “physiological insulin resistance”.

Whether a more sustained decrease in AMPK activity caused by glucose and leucine can lead to pathological insulin resistance (ie insulin-resistance associated with less reversible cellular abnormalities) in vivo remains to be determined. In keeping with such a notion, the insulin resistance caused by exposure of cultured endothelial cells to a high glucose medium for 24 h is associated with mitochondrial dysfunction, apoptosis, oxidative stress and inflammation (33-35), all of which are prevented by AMPK activation. The early observation that amino acid levels are elevated in the plasma of obese individuals (36, 37) first raised the possibility that they may be involved in the development of obesity-linked and/or diet-induced insulin resistance. Subsequently, infusion of the branched-chain amino acid leucine was found to impair glucose uptake in humans despite elevated plasma insulin levels (38) and in rodents fed a high fat diet, branched chain amino acids have been implicated in the development of obesity-associated insulin resistance (39). Whether decreases in AMPK activity or an impairment of its activation occurred in these settings was not examined. Finally, decreased AMPK activity has been observed in many rodents with chronic insulin resistance including ob/ob mice (40), fa/fa and ZDF rats (41, 42) and the IL-6 KO mouse (43). However, in all of these rodents it was attributable to a genetic lack of a hormone or its receptor.

In summary, we have demonstrated that incubation of rat skeletal muscle with moderately elevated concentrations of leucine or glucose suppresses AMPK activation and concomitantly increases mTOR/p70S6K signaling, protein synthesis and leads to insulin resistance. All of these changes were associated with a decrease in AMPK activity and were prevented by incubating the muscles with the AMPK activators AICAR or α-lipoic acid. Although collectively these findings suggests that a decrease in AMPK activity mediates the effects of high glucose and
leucine, direct evidence for this is still lacking.

**Author contributions.** X. J. X., E.L, R.D. researched data. A.K.S. wrote manuscript, contributed discussion, reviewed/edited manuscript. A.E.B researched data and reviewed/edited manuscript. E.W.K. contributed to discussion, reviewed/edited manuscript and N.B.R. wrote manuscript, contributed to discussion, reviewed/edited manuscript.

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**REFERENCES:**


Table 1. Effects of leucine and high glucose on adenine nucleotide.

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<th>ATP</th>
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<th>CrP</th>
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<tr>
<td>(5 mM glucose)</td>
<td>3.9±0.01</td>
<td>0.04±0.001</td>
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<td>13±2</td>
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<td><strong>Leucine</strong></td>
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<td>(100 μM)</td>
<td>3.8±0.01</td>
<td>0.035±0.001</td>
<td>0.04±0.001</td>
<td>14.5±4</td>
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<td><strong>Glucose</strong></td>
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<tr>
<td>(25 mM)</td>
<td>4.2±0.2</td>
<td>0.045±0.002</td>
<td>0.6±0.03</td>
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Table 2. Effects of leucine and high glucose on lactate and pyruvate.

<table>
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<th>Lactate (L)</th>
<th>Pyruvate (P)</th>
<th>L/P</th>
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<tr>
<td>(5mM glucose)</td>
<td>12±2</td>
<td>1.1±0.1</td>
<td>11±2</td>
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<td><strong>Leucine</strong></td>
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<tr>
<td>(100 μM)</td>
<td>29±4*</td>
<td>1.4±0.1*</td>
<td>21±3*</td>
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<tr>
<td><strong>Glucose</strong></td>
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<td>(25 mM)</td>
<td>30±2*</td>
<td>1.7±0.2*</td>
<td>18±1.5*</td>
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Nucleotide values were expressed as mmol/g muscle. Lactate and pyruvate were Expressed as μmol/mg muscle. Results are means±SE (n=5-6). *Significantly different from control at 5 mM glucose (p<0.05).

**FIGURE LEGENDS**

**FIGURE 1. Effect of incubation with different concentrations of leucine on protein synthesis and p-mTOR, P-p70S6K and p-4EBP1 phosphorylation.** EDL were incubated in Krebs-Henseleit solution containing 0, 100 and 200 μM of leucine for 1hr. Protein synthesis was determined, at the end of the incubation, on the basis of phenylalanine incorporation into protein (A) and the phosphorylation of mTOR, p70 S6 kinase and 4EBP1 by immunoblot analysis. Representative western blots (B) and densitometric analysis (C). Results are means ± SE, (n = 6); *, p<0.05 relative to no leucine or 0 time.

**FIGURE 2. Dose response and time-course of the effects of leucine on AMPK phosphorylation and activity.** EDL were incubated in the absence (0) or presence (50, 100 or 200 μM) of leucine for 1h and muscle lysates analyzed for (A) the phosphorylation of AMPK
and its downstream target P-ACC using SDS-PAGE and (B) α2-AMPK activity. (C) the time course (15-120 min) of changes in the abundance of P-AMPK in muscles incubated with 100 μM leucine. (D) comparison of effects of leucine, isoleucine and glutamine (100 μM) on total AMPK and P-AMPK. Results are means ± SE, (n = 6); *, p<0.05 relative to 0 μM of leucine or 0 time.

**FIGURE 3. Effect of incubation with different concentrations of glucose on protein synthesis, mTOR, p70S6 kinase and 4-EBP1 phosphorylation.** EDL were incubated for 1 h in media containing 0, 5.5 or 25 mM glucose. (A) Protein synthesis (B) Representative western blots for P-mTOR, P-p70S6 kinase and P-4EBP1 (top). Quantification of western blots (bottom). Results are means ± SE, (n = 4); *, p<0.05 vs 0 glucose and **, p<0.01 vs 0 and 5.5 mM glucose.

**FIGURE 4. Effect of incubation with different concentrations of glucose on AMPK and ACC phosphorylation and AMPK abundance and on malonyl CoA content.** EDL were incubated for 1 h in media containing 0, 5.5 or 25 mM glucose. Representative western blots (upper panel). Densitometric analysis of blots and malonyl CoA content (lower panel). Results are means ± SE, (n = 4); *, p<0.05 vs 0 mM glucose and **, p<0.01 vs 0 and 5.5 mM glucose.

**FIGURE 5. Effect of AICAR on protein synthesis, AMPK α1 and α2 activity and phosphorylation, mTOR, p70S6K and ACC phosphorylation and malonyl CoA content.** EDL were preincubated with Krebs-Henseleit solution containing 5.5 mM glucose for 20 min and then for an additional 1 h in presence of 1 mM AICAR. Protein synthesis was measured at 0 or 5.5 mM glucose or 5.5 mM glucose and AICAR (1 mM) (A), muscle supernatants were subjected to immunoprecipitation with specific antibodies to the α1 and α2 of AMPK (B) and AMPK (panel B), ACC (C), mTOR and p70S6K phosphorylation (D) were determined. malonyl CoA concentration (C) were determined as described in the Methods. See Figures 3 and 4 for P-AMPK, P-ACC, P-mTOR and P-p70S6 K at 0 glucose. Results are means ± SE of three experiments *, p<0.05 vs 5.5 mM glucose. *, p<0.01 vs 5.5 mM glucose.

**FIGURE 6. Comparison of effect of AICAR (1 mM) on AMPK and mTOR phosphorylation and protein synthesis in muscles incubated with 5 or 25 mM glucose or leucine.** EDL were preincubated with Krebs-Henseleit solution containing 5.5, 25 mM glucose or 100 μM leucine for 30 min and then additional 1 h in the presence or absence of AICAR (1 mM). Results are means ± SE of four experiments *, p<0.05 vs 5 mM glucose. ** p<0.01 vs compared to 5 mM glucose alone. # p<0.05 vs 5 mM glucose plus AICAR.

**FIGURE 7. AICAR prevents the inhibition of insulin-stimulated PKB/Akt phosphorylation caused by leucine** EDL were preincubated with leucine (100 μM) for 30 min and then AICAR for 1hr. They were then incubated with insulin (10mU/ml) for 10 min. Muscle lysates were analyzed for PKB/Akt using SDS-PAGE and quantified as described in the Methods. Results are means ± SE, (n = 6). *P<0.05 vs basal, **p<0.01 vs insulin and #p<0.01 vs insulin+leucine.

**FIGURE 8. AMPK α2 activity and phosphorylation of mTOR and p70 S6 kinase following glucose infusion.** Red gastrocnemius muscle was frozen in liquid nitrogen after 0 or 5 h of glucose infusion and α2-AMPK activity and phosphorylation of mTOR and p70 S6K were determined as described in the Methods. Results are means±SE (n=4-6 rats per group). *p<0.05 vs basal.
AMPK and protein synthesis in skeletal muscle

Figure 3

A

![Bar graph showing protein synthesis in response to glucose concentration (mM)]

B

![Western blot analysis of protein expression with glucose concentration (mM)]

Figure 4

![Western blot analysis of AMPK, ACC, P-AMPK, and P-ACC with glucose concentration (mM)]
AMPK and protein synthesis in skeletal muscle

Figure 5

A

Protein Synthesis (nmol Phe/mg protein/h)

<table>
<thead>
<tr>
<th></th>
<th>Glucose (0 mM)</th>
<th>Glucose (5.5 mM)</th>
<th>AICAR (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AMPK</td>
<td></td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>AMPK</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

AMPK activity (nmol/min/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AICAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AMPK in EDL muscle</td>
<td>1500</td>
<td>500</td>
</tr>
<tr>
<td>AMPK phosphorylation</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

C

Malonyl CoA (nmol/mg muscle)

<table>
<thead>
<tr>
<th></th>
<th>Glucose (0 mM)</th>
<th>Glucose (5.5 mM)</th>
<th>AICAR (1 mM)</th>
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<tbody>
<tr>
<td>ACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-ACC</td>
<td></td>
<td>*</td>
<td>**</td>
</tr>
</tbody>
</table>

D

P-mTOR

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AICAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-p70S6K</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>mTOR</td>
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</tbody>
</table>

Figure 6

A

P-AMPK

B

P-mTOR

C

P-AMPK/AMPK (AU)

<table>
<thead>
<tr>
<th></th>
<th>5.5 mM glucose</th>
<th>25 mM glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>AICAR</td>
<td></td>
<td>400</td>
</tr>
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</table>

D

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Leucine</th>
<th>Basal + AICAR</th>
<th>Leucine + AICAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AMPK/AMPK (AU)</td>
<td>945 ±52</td>
<td>250 ±20</td>
<td>1650 ±125</td>
<td>924 ±30</td>
</tr>
<tr>
<td>P-mTOR/mTOR (AU)</td>
<td>552 ±65</td>
<td>900 ±45</td>
<td>221 ±25</td>
<td>500 ±40</td>
</tr>
<tr>
<td>Protein synthesis (nmol Phe/mg protein/h)</td>
<td>1.2 ±02</td>
<td>2 ±0.5</td>
<td>0.7 ±0.6</td>
<td>1.1 ±0.4</td>
</tr>
</tbody>
</table>
Figure 7

**P-AKT**\(_{\text{Ser }473}\) /**total AKT** (AU)

<table>
<thead>
<tr>
<th>Glucose (5 mM)</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin</strong> (10mU/ml)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Leucine</strong> (100(\mu)M)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>AICAR</strong> (1 mM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 8

A

AMPK activity (nmol/min/g protein)

Basal 5h

B

P-70S6K (AU)

Basal 5h

C

P-mTOR (AU)

Basal 5h

* Indicates significant difference from Basal.