Lipid-induced insulin resistance is prevented in lean and obese myotubes by AICAR treatment

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Bikman BT, Zheng D, Reed MA, Hickner RC, Houmard JA, Dohm GL. Lipid-induced insulin resistance is prevented in lean and obese myotubes by AICAR treatment. Am J Physiol Regul Integr Comp Physiol 298: R1692–R1699, 2010. First published April 12, 2010; doi:10.1152/ajpregu.00190.2009.—The molecular mechanisms of obesity-associated insulin resistance are becoming increasingly clear, and the effects of various lipid molecules, such as diacylglycerol and ceramide, on the insulin signal are being actively explored. To better understand the divergent response to lipid exposure between lean and obese, we incubated primary human muscle cells from lean [body mass index (BMI) <25 kg/m²] and morbidly obese (BMI >40 kg/m²) subjects with the saturated fatty acid palmitate. Additionally, given that AMPK-activating drugs are widely prescribed for their insulin-sensitizing effects, we sought to determine whether 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR)-stimulated AMPK activation could prevent or reverse the deleterious effects of lipid on insulin signaling. We found that a 1-h palmitate incubation in lean myotubes reduced (P < 0.05) insulin-stimulated phosphoprotein kinase B (Akt), Akt substrate 160 (AS160), and inhibitory factor kB (IκBα) mass, all of which were prevented with AICAR inclusion. With a longer incubation, we observed that myotubes from morbidly obese individuals appear to be largely resistant to the detrimental effects of 16 h lipid exposure as was evident, in contrast to the lean, by the absence of a reduction in insulin-stimulated insulin receptor substrate (IRS)-1 Tyr phosphorylation, phospho-Akt, and phospho-AS160 (P < 0.05). Furthermore, 16 h lipid exposure significantly reduced IκBα levels and increased phosphorylation of c-Jun NH2-terminal kinase (JNK) and IRS1-Ser312 in lean myotubes only (P < 0.05). Despite a divergent response to lipid between lean and obese myotubes, AICAR inclusion improved insulin signaling in all myotubes. These findings suggest an important role for regular exercise in addition to offering a potential mechanism of action for oral AMPK-activating agents, such as thiazolidinediones and metformin.

5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside; adenosine 5'-monophosphate-activated protein kinase; inhibitor of κB kinase; c-Jun amino-terminal kinase; skeletal muscle

SKELETAL MUSCLE IS THE MAIN site of insulin-mediated glucose uptake, and it has been shown that skeletal muscle is the principal site for peripheral insulin resistance (43). Moreover, it is well established that intramyocellular lipid accumulation is associated with insulin resistance (35). Our group has previously demonstrated that obesity is associated with reduced insulin-stimulated glucose uptake (12) and that weight loss, through both lifestyle intervention (28) and surgery (5, 21, 29), reduces intramyocellular lipid and improves insulin sensitivity in humans. Additionally, lipid metabolites such as ceramide and diacylglycerol (DAG) have been implicated to inhibit insulin signaling in a variety of tissue, including liver (31) and skeletal muscle (9, 53).

The mechanisms through which lipids interrupt the insulin signaling cascade are becoming increasingly clearer, with a great deal of attention being placed on the differing ends of the insulin signaling pathway. Distally, the sphingolipid ceramide impedes insulin action by inhibiting protein kinase B (Akt) phosphorylation, a distal and necessary step preceding GLUT4 translocation. This is thought to largely occur via the dephosphorylation of Akt by ceramide-dependent activation of protein phosphatase 2A (46). Proximally, serine phosphorylation of insulin receptor substrate (IRS)-1 (8, 53) by lipid-sensitive serine kinases such as the novel protein kinase C (PKC) isoforms (22, 42, 53), c-Jun NH2-terminal kinase (JNK) (1, 11, 41), and the inhibitor of κB kinase β (IKKβ) (2, 8, 30, 42, 44, 53, 54), has been found to inhibit insulin signal transduction (8, 54), and the IRS-1 Ser312 (Human/J307/rodent) residue has received particular interest given its proximity to the phosphotyrosine-binding domain in IRS-1. IRS-1 undergoes a conformational change as a result of serine phosphorylation that decreases its ability to interact with the insulin receptor, making it a poor substrate for the insulin receptor (1). Both obesity and insulin resistance are associated with an increased low-grade inflammatory tone, as evidenced by increased levels of proinflammatory cytokines as well as greater activity of the inflammatory factor, NF-κB (4, 16, 37, 50), which is responsible for the transcription of multiple cytokines (including TNF-α and IL-1β) (20, 40). IKKβ is also a prominent mediator in the NF-κB pathway through its actions on the NF-κB inhibitor IκBα. Briefly, IκBα is phosphorylated by IKKβ, which results in the rapid degradation of IκBα and subsequent migration of NF-κB into the nucleus.

A host of insulin-sensitizing drugs have been shown to act, in part, through inhibition of NF-κB pathway activity, including salicylate (30), thiazolidinediones (TZDs) (18, 32, 48), and metformin (10, 55). Interestingly, recent evidence indicates that both TZDs and metformin increase activity of the AMP-activated protein kinase (AMPK). AMPK is ubiquitous in mammalian cells and is considered the “fuel gauge” of the cell, responding when cellular energy status is threatened, such as a decrease in ATP concentration, by induction of catabolic ATP-generating processes and inhibition of synthetic (ATP-consuming) processes (52). By stimulating the mitochondrial import and oxidation of fatty acids by inhibiting carnitine palmitoyl transferase-1 (15, 35), AMPK likely prevents the accumulation of lipid metabolites that activate NF-κB pathway intermediates and disrupt insulin signal transduction (3, 14). However, AMPK has also been shown to prevent cytokine-
induced NF-κB pathway stimulation and result in reduced IKKβ action (7, 10, 23, 24, 33), suggesting a more direct effect of AMPK on the NF-κB pathway.

In the present study, we more deeply explore the result of various palmitate incubation periods in human myotubes from lean and obese subjects in regard to its effect on insulin signaling. Additionally, given the widely prescribed use of oral AMPK activators in diabetes treatment, we examine whether 5-aminoimidazole-4-carboxamide 1-β-d-ribofuranoside (AICAR)-induced AMPK activation mitigates and/or prevents the deleterious effects of lipid on insulin signaling.

MATERIALS AND METHODS

Subjects. Lean (n = 8; BMI <25 kg/m²) and morbidly obese (n = 8; BMI >40 kg/m²) Caucasian women were recruited for participation in the study. All subjects were considered sedentary, weight stable, and in good health after filling out a medical history questionnaire. None suffered from overt metabolic disease, such as type 2 diabetes mellitus. Subjects were informed of potential risks associated with the muscle biopsy and signed an informed consent document before biopsies were taken. The protocol and consent form were approved by the East Carolina University Institutional Review Board.

Primary human muscle cell cultures. Following a 12-h overnight fast, skeletal muscle from the vastus lateralis of subjects was obtained by percutaneous biopsy (13). Approximately 40–50 mg of muscle sample was transferred to refrigerated low-glucose Dulbecco’s modified Eagle’s medium [DMEM; media and media supplements were purchased from GIBCO (Invitrogen, Carlsbad, CA) unless otherwise stated], and all visible connective and adipose tissue was removed before culture. Satellite cells were isolated by trypsin digestion, preplated 1–3 h in 3.0 ml of growth media (GM) [DMEM supplemented with 10% FBS, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, 20 ng/ml human epidermal growth factor, 0.39 μg/ml dexamethasone, and 50 μg/ml gentamicin/amphotericin B (Lonza, Walkersville, MD)] on an uncoated T-25 tissue culture flask (Fisher Scientific, Waltham, MA) to remove fibroblasts, and then transferred to a type I collagen-coated T-25 flask for myoblast attachment. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. After reaching ~70% confluence, myoblasts from lean and obese subjects were separately pooled by adding roughly 100 × 10⁶ cells/sample in a lean or obese cell pool, respectively. After achieving ~90% confluence, cells were differentiated by replacing GM with low-serum differentiation media (DMEM, 2% heat-inactivated horse serum, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, and 50 μg/ml gentamicin/amphotericin B). Media was changed every 1–2 days. Media-containing lipid was prepared by dissolving palmitate in ethanol and diluting in DMEM with 1% BSA and 1 mM carnitine. On the treatment day (day 6), mature myotubes were serum starved with fasting media (DMEM, 0.2% BSA) for 4 h before lipid treatment.

The initial experiment involved myotubes from the lean cell bank exposed to 1-h incubations with 0.45 mM palmitate in the presence or absence of 2 mM AICAR (Calbiochem, San Diego, CA). The secondary experiment involved myotubes from lean and obese cell banks exposed to a 16-h incubation in one of two conditions using DMEM with 1% BSA and 1) no supplementation or 2) supplemented with 0.45 mM palmitate. The final experiment similarly employed myotubes from lean and obese cell banks. Following serum starvation, cells were incubated for 16 h in one of four conditions using DMEM with 1% BSA and 1) no supplementation (control), 2) supplemented with 0.45 mM palmitate, 3) supplemented with 0.45 mM palmitate and 2 mM AICAR, and 4) supplemented with 0.45 mM palmitate with 2 mM AICAR added for the final 4 h. During the final 10 min of treatment, cells were incubated in the presence or absence of 100 nM insulin and subsequently rinsed with PBS, harvested in 400 μl of ice-cold lysis buffer [50 mM HEPES, 100 mM sodium fluoride, 50 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 10 mM EDTA, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO)] per well, and sonicated for 5 s, followed by a 2-h rotation at 4°C. Cell viability was determined by comparison with control before and after treatment by trypan blue cell exclusion.

Immunoblotting. Total protein content was measured using a BCA protein assay (Pierce, Rockford, IL), and individual homogenate volumes were aliquoted into 20 and 200 μg of protein before being frozen in liquid nitrogen and stored at −80°C until used for immunoblotting. For IRS-1, IRS1-phospho (p)-Ser112, IRS1-phosphoryrosine, and Akt substrate 160 (AS160), 200 μg homogenates were subjected to 10 μl IRS-1 monoclonal immunoprecipitated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 10 μl AS160 polyclonal immunoprecipitated antibody (Cell Signaling, Beverly, MA), respectively, overnight and then coupled to protein A sepharose beads (Amersham Biosciences, Uppsala, Sweden) for 12 h. pSer112, and AS160) or to 40 μl anti-phosphotyrosine-agarose antibody (Sigma-Aldrich), respectively, then rotated for 2 h at 4°C and eluted with a 1:10 solution of 2× Breaker TCEP (Pierce) and Laemmli Sample Buffer (Bio-Rad, Hercules, CA). Samples were separated by SDS-PAGE using 7.5 or 10% Tris-HCl gels and then transferred to polyvinyliden fluoride membranes for probing by appropriate antibodies. Following incubation with primary antibodies, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. IRS-1 and pIRS1 Ser307 antibodies were purchased from Millipore (Billerica, MA). Antibodies for pAMPKα (Thr172), AMPKα, p-acetyl-CoA carboxylase (ACC), ACC, pAkt-substrate, and pan-AS160 were purchased from Cell Signaling Technologies (Beverly, MA), and pAkt, Akt, IκBα, pINK (Thr183/Tyr185), JNK1, and actin were from Santa Cruz Biotechnology. Horseradish peroxidase activity was assessed with enhanced chemiluminescence solution (Thermo Scientific, Rockford, IL) and exposed to film. The image was scanned, and band densitometry was assessed with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Equal loading of proteins was ensured by probing for actin. Content of phosphoproteins (using phosphospecific antibodies) was calculated from the density of the band of the phosphoprotein divided by the density of the total protein using the appropriate antibody.

Statistics. Data are expressed as means ± SE of at least four separate experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey’s post hoc test where necessary. Significance was set at P < 0.05.

RESULTS

Treatment in lean myotubes for 1 h. In control media, insulin stimulation was associated with reduced pAMPK levels (P < 0.05; Fig. 1A), a finding confirmed in other studies (14). Additionally, 1 h of lipid incubation had no significant effect on pAMPK levels or its substrate, pACC (Fig. 1B). In contrast, when AICAR was included in the incubation, phosphorylation of both AMPK and ACC increased dramatically (P < 0.001). As expected, insulin stimulation significantly elevated Akt phosphorylation and, in contrast to AMPK, insulin-stimulated pAkt was significantly diminished with lipid incubation (P < 0.001; Fig. 1C), and AICAR coinoculation prevented this effect. A similar trend was observed with AS160 activation; lipid incubation significantly reduced insulin-stimulated AS160 phosphorylation (P < 0.001) and AICAR inclusion restored this effect (Fig. 1D). Additionally, AICAR significantly elevated basal pAS160 levels (P < 0.05), which supports evidence indicating AMPK’s action on AS160. Inasmuch as IKKβ has been implicated in reducing insulin signaling through its inhibitory actions on IRS-1, IκBα, a downstream substrate and inverse indicator of IKKβ, was determined as a
measure of IKKβ action, and JNK phosphorylation was determined. Lipid treatment significantly reduced IκBα mass (P < 0.05), although this effect was prevented with AICAR coincubation (Fig. 1E). Moreover, although there was a tendency for insulin stimulation to reduce IκBα in control media, this disparity increased with lipid treatment (P < 0.05), suggesting an unexpected synergistic action of lipid and insulin on reducing IκBα mass. In contrast, palmitate had no effect on JNK activation with the 1-h treatment (data not shown). Furthermore, insulin stimulation had no significant effect on IRS1-Ser312 phosphorylation in lean myotubes in control media, although palmitate incubation was associated with elevated levels of insulin-stimulated IRS1-pSer312 (P < 0.05; Fig. 1F). Conversely, AICAR inclusion significantly reduced Ser312 phosphorylation in both basal and insulin-stimulated cells compared with both control and lipid incubation alone (P < 0.01).

Lipid treatment in lean and obese myotubes for 16 h. We were further interested in comparing myotubes from lean and obese subjects to explore whether they exhibit a divergent response to palmitate incubation. First, no baseline differences in pAMPK or pACC were observed between lean and obese cells, and lipid incubation appeared to have no effect on either protein (data not shown). As a marker of the proximal insulin signal, IRS-1 tyrosine phosphorylation was determined. Insulin increased IRS1-pTyr levels in lean cells (P < 0.01), but not obese in control media, and insulin-stimulated IRS1-pTyr levels were greater in lean myotubes compared with obese (P < 0.01; Fig. 2A). However, lipid treatment significantly reduced the insulin-stimulated IRS-1 tyrosine phosphorylation in the myotubes from lean subjects (P < 0.05), with no effect on those from obese subjects.

In exploring downstream markers of insulin signaling, phosphorylation states of both Akt and AS160 were determined. Insulin-stimulated pAkt was significantly elevated in all treatments in both lean and obese myotubes (Fig. 2B). Interestingly, this effect was diminished with lipid incubation in lean myotubes only. In fact, the difference in insulin-stimulated pAkt levels between the lean and obese myotubes in control media (P < 0.05) were removed upon inclusion of palmitate such that there was no significant difference between the two when lipid was included in the incubation. Furthermore, pAS160 levels in the lean myotubes followed a similar pattern to pAkt in that insulin resulted in pAS160 increasing significantly (P < 0.01) as well as the effect of palmitate in reducing the magnitude of change (Fig. 2C). In contrast, insulin stimulation had no effect on pAS160 levels in myotubes from obese subjects despite differences in pAkt, suggesting an interruption between Akt and AS160 in the obese subjects.
Baseline differences in IκBα mass were observed between myotubes from lean and obese subjects (P < 0.01; Fig. 2D), suggesting that differences observed in vivo are retained in culture (5). Additionally, lipid treatment was associated with reduced basal IκBα levels in lean myotubes, and the disparity between basal and insulin-stimulated levels increased with lipid incubation (P < 0.05). As seen with the 1-h treatment, IκBα levels were reduced in the presence of insulin and lipid together (P < 0.05). JNK phosphorylation significantly increased in myotubes from lean subjects after 16 h of palmitate incubation (P < 0.05), whereas myotubes from the obese experienced no change, strengthening the observation that obese muscle cells may be somewhat resistant to the consequences of lipid incubation (Fig. 2E).

Lipid and AICAR treatment in lean and obese myotubes for 16 h. With the disparate responses to lipid exposure between lean and obese myotubes being observed, a follow-up experiment was performed to determine whether AICAR-induced AMPK activation prevents the decrease in insulin signaling with long-term lipid exposure and whether lean and obese myotubes respond differently to AICAR. When AICAR was included in the palmitate incubation for either the final 4 h or the full 16-h treatment period, a robust increase in pAMPK and pACC levels was observed compared with lipid treatment alone in both lean and obese myotubes (P < 0.001; Fig. 3, A and B). AMPK activation with AICAR significantly elevated insulin-stimulated IRS1-pTyr in the lean (P < 0.001) myotubes compared with lipid alone, but the tendency for improvement with the AICAR inclusion in the obese myotubes did not reach significance (Fig. 3C).

In looking downstream, insulin-stimulated pAkt increased with AICAR inclusion at both time points in the lean and obese myotubes (P < 0.01; Fig. 3D). In contrast, pAS160 levels only tended to increase with the 4-h AICAR incubation in both sets of myotubes (Fig. 3E). Nevertheless, full coincubation with both lipid and AICAR significantly increased pAS160 levels in lean and obese vs. lipid alone (P < 0.05), although insulin-stimulated pAS160 levels in obese myotubes were lower compared with lean in the same treatment (P < 0.05). The lack of change with insulin stimulation of AS160 is likely explained by AMPK’s ability to independently activate AS160.

IκBα levels were similar between lean and obese myotubes when incubated with lipid alone (P < 0.05; Fig. 3F). At both 4 and 16 h of AICAR incubation with lipid, IκBα increased in lean (P < 0.01) and obese (P < 0.05) subjects, although the effect was not as robust in the obese myotubes, and a difference was observed compared with lean subjects (P < 0.05). In contrast, AICAR incubation reduced pJNK in lean myotubes.
exclusively \((P < 0.05)\), with no effect in the obese culture (Fig. 3G). Additionally, AICAR treatment significantly reduced the increase in IRS1-Ser\(^{312}\) phosphorylation induced by lipid incubation in insulin-stimulated cells in all cells \((P < 0.05\); Fig. 3H\).

DISCUSSION

The findings from the current study support the mounting evidence that saturated fatty acids induce insulin resistance in skeletal muscle via decay of the insulin signal. Specifically, we found that a 1-h incubation of the saturated fatty acid palmitate with myotubes from lean humans elicited a significant reduction in insulin-stimulated \(\text{pAkt and } I_s^B\) mass as well as an elevation in IRS-1 Ser\(^{312}\) phosphorylation and that the effects of lipid incubation were completely prevented with AICAR inclusion. Moreover, we observed divergent responses between the lean and obese myotubes as was evident in the 16 h lipid treatment by the absence of a reduction in insulin-stimulated IRS-1 Tyr phosphorylation, levels of \(\text{pAkt and pAS160}\). Furthermore, whereas lipid exposure significantly reduced \(I_s^B\) levels and increased IRS1-pSer\(^{312}\) in lean myotubes, no effect was observed in the obese. However, despite a divergent response to lipid between lean and obese myotubes, AICAR inclusion promiscuously restored insulin signaling at every measured step in the lean and improved the signal in obese myotubes. The lack of an observed response to lipid treatment in the obese myotubes is likely a result of the chronically

Fig. 3. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI <25 m/kg\(^2\)) and morbidly obese (BMI >40 m/kg\(^2\)) humans. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4 h AICAR (gray bars), or lipid + 16 h AICAR (black bars) in the presence or absence of 10 min insulin (100 nM) and probed for \(\text{pAMPK (A), pACC (B), IRS1-pTyr (C), pAkt (D), pAS160 (E), I_s^B (F), pJNK (G), and IRS1-pSer}^{312}(H)\). \(P < 0.05\) for basal vs. insulin stimulation within a given treatment (*), coincubations (4 and 16 h) vs. lipid alone within a given group (#), and obese vs. lean within a given treatment (•).
elevated intracellular and circulating lipid levels seen with obesity. Indeed, it is possible that the apparent lipid-resistant state of the obese myotubes is due to the fact that they “responded” to the chronically high lipid environment long ago.

Recent advances in the area of obesity and diabetes research have revealed a role for various lipid-stimulated kinases that act at IRS-1 and result in attenuation of the insulin signal, namely IKKβ, PKCθ, and JNK1 (27). We previously reported differences in IκBα protein levels, an inverse measure of IKKβ action, and IRS1-Ser312 phosphorylation in vastus lateralis of lean and morbidly obese humans (5) and herein report retention of this disparity in cultured myotubes. Moreover, in the lipid- and insulin-sensitive lean myotubes, we observed a significant decrease in IκBα levels when exposed to lipid and insulin concurrently in both the 1- and 16-h treatments. Lipid-induced reductions in IκBα levels have been observed as early as 30 min after lipid inclusion (51), and our findings of a decrease in IκBα after 10 min were somewhat unexpected. However, a key difference in the current study is the presence of insulin. Insulin’s anabolic role encourages the rapid influx and storage of fatty acids in skeletal muscle (39), and the decrement in IκBα after 10 min of insulin treatment in the current study may be a function of the synergistic effects of lipid and insulin, resulting in a rapid uptake of lipid in the absence of a comparable increase in oxidation. Similarly, it is noteworthy that we did not observe an increase in AMPK phosphorylation with palmitate incubation, whereas others have noticed such an effect (14, 38). We believe this discrepancy is likely due to the different origins of the myotubes, primary human cells vs. immortalized rat muscle cells.

In support for our findings implicating a role for IKKβ in mediating lipid-induced insulin resistance in muscle, Sinha et al. (44) observed that 6 h of palmitate exposure in L6 myotubes resulted in a significant decrease in insulin-stimulated glucose uptake, as well as reduced IκBα mass and pAkt levels. However, insulin signaling was restored with salicylate treatment. Additionally, salicylate treatment in rodents has been shown to be associated with improved glucose tolerance and improved lipid profile (30, 54). Also, heterozygous IKKβ+/− mice fed a high-fat diet have been found to have lower fasting insulin and glucose values compared with IκBα−/− littermates (54). Furthermore, Kim et al. (30) observed that IKKβ knockout mice did not display any decrement in skeletal muscle insulin signaling with lipid infusion.

In addition to our observations regarding IκBα, we explored the role of JNK1 in mediating and preventing the lipid-induced decay in the insulin signal. Similar to IKKβ, JNK1 is an inflammatory serine kinase reportedly involved in inducing serine phosphorylation of IRS-1 in response to lipid (25, 34). We found that a 1-h palmitate treatment was insufficient to activate JNK in lean myotubes and that AICAR inclusion at this time point similarly had no effect. However, a 16-h palmitate incubation stimulated JNK phosphorylation, and AICAR treatment inhibited lipid-induced JNK phosphorylation in lean myotubes exclusively. Interestingly, JNK phosphorylation did not change with any treatment in myotubes from obese subjects. The disparate responses of IκBα and JNK to palmitate regarding incubation time and cell source (obese vs. lean) were unexpected. Regarding timing, although IκBα was apparently affected with both lipid and lipid + AICAR in lean myotubes at 1 h, JNK responded to neither treatment, whereas, at 16 h, palmitate significantly reduced IκBα levels and induced JNK activation in myotubes from lean subjects, a finding that was supported with an almost twofold increase in IRS1-pSer312 levels. Similarly, IRS1-pSer312 levels measured in myotubes from obese at 16 h followed the trend of both IκBα and JNK in these myotubes; no change was observed.

Despite the evident lipid resistance of the myotubes from the obese subjects, these cells were nonetheless responsive to AICAR with an approximately twofold increase in IκBα levels over lipid alone. Whereas AICAR inclusion appeared to reduce JNK phosphorylation in the obese myotubes, this trend did not reach significance. Subsequently, whereas the reduction in IRS1-Ser312 phosphorylation (~70%) in the lean myotubes with AICAR at both time points can be explained by a reduction in both IKKβ action on IκBα and JNK activation, the approximate 40% change in IRS1-Ser312 phosphorylation in obese myotubes with AICAR inclusion appears to largely be a result of reduced IKKβ action alone.

Regarding the negative impact of lipids on insulin signal transduction, bioactive lipids appear to burn the candle at both ends, with lipid-sensitive serine kinases (JNK, IKKβ, and PKC) implicated in checking IRS-1 function at the signal’s origin and sphingolipid-mediated inhibition in Akt activation affecting its conclusion. Although initial research into insulin signaling focused predominantly on IRS-1 function, a more recent surge of work has begun to establish that factors affecting the distal end of insulin signal transduction have a greater impact on glucose uptake compared with those acting more proximally. In particular, work by Ng et al. (36) has established that activation of Akt2 in 3T3-L1 adipocytes is sufficient to induce glucose uptake in the absence of IRS-1 activation and that GLUT4 translocation and glucose uptake appear to be unaffected by modulations in IRS-1. With these observations in mind, it is important to note the limitation of the current study in that glucose uptake was not determined.

Regarding the distal portion of the insulin signal, we observed that lipid incubation significantly reduced the ability of insulin to stimulate Akt phosphorylation in the lean subjects. However, compared with lipid alone, AICAR dramatically increased insulin-stimulated pAkt in both lean and obese subjects. AMPK has been shown to induce glucose uptake through activation of AS160, which is downstream of Akt (6, 47, 49). Additionally, the observation that AICAR had no effect on basal Akt phosphorylation suggests, in contrast to AS160 phosphorylation, that AMPK is not acting directly on Akt, but is rather facilitating the ability of insulin to stimulate Akt phosphorylation. With this in mind, it is tempting to speculate that AICAR-stimulated AMPK activation attenuates the lipid-induced decrement in upstream insulin signaling. Research in various cell types has revealed a role for AMPK in inhibiting NF-κB pathway activity (7, 19, 23), although evidence of this effect in skeletal muscle is scarce and contrasting (26, 33, 45).

Considering that AICAR treatment in human myotubes has also been found to decrease cytokine production (33), we feel that these observations, in addition to our own findings that AICAR treatment restores IκBα levels in the lean and elevates levels above normal in the morbidly obese, suggest a potential role for AMPK in preventing the actions of lipid-sensitive kinases on IRS-1 function.
As mentioned, in exploring the distal insulin signal, we observed an unexpected incongruity between Akt and AS160 phosphorylation. AICAR treatment increased insulin-stimulated Akt phosphorylation in both basal and insulin-stimulated myotubes at both 4 and 16 h of incubation. However, AICAR treatment elicited an increase in pAS160 even in the absence of insulin, offering support for AMPK’s direct action on AS160 (47). In addition, we observed that AICAR treatment only increased pAS160 with full AICAR + lipid coinoculation and that inclusion of AICAR in the final 4 h of treatment elicited essentially no effect. This difference may represent an inability of AMPK activation to fully restore the decay of the insulin signal following a prolonged period of lipid incubation, whereas AICAR inclusion at the onset of lipid incubation can prevent this decay. The ability of AMPK action to prevent lipid-induced insulin resistance has been recently explored in skeletal muscle. Namely, Fujii et al. (17) observed reduced insulin receptor-β subunit, IRS-1, and Akt in muscle-specific transgenic mice expressing an inactive form of AMPKα2, whereas muscle levels of DAG did not differ between the mice on control diet, DAG levels were significantly elevated in transgenic mice vs. control when fed a high-fat diet. Furthermore, Akt activity and phosphorylation of IRS1-Tyr, Akt, and AS160, and elevated DAG levels were significantly elevated in transgenic mice vs. control when fed a high-fat diet. These observations correlated with a reduction in insulin-stimulated glucose uptake in isolated soleus of transgenic mice.

**Perspectives and Significance**

In summary, the present study shows a divergent response to palmitate incubation between myotubes from lean and morbidly obese humans. Palmitate incubation in lean myotubes was associated with reduced IκBα protein mass, reduced phosphorylation of IRS1-Tyr, Akt, and AS160, and elevated IRS-1 Ser112 phosphorylation, whereas the myotubes from the morbidly obese did not appear to be significantly affected. In AICAR + lipid coinoculation, AICAR treatment prevented the decay in insulin signaling observed with lipid alone, although AICAR inclusion in the final 4 h of lipid incubation was not sufficient to restore AS160 phosphorylation. Additionally, AICAR-induced AMPK activation was associated with elevated IκBα and reduced JNK phosphorylation in the lipid-sensitive myotubes from lean subjects. We conclude that AICAR-induced AMPK activation prevents lipid-induced insulin resistance in lean myotubes and restores signaling in obese myotubes to previously unseen levels through the inhibition of lipid-stimulated serine kinases. These findings suggest an important role for regular exercise in addition to offering a potential mechanism of action for oral AMPK-activating agents, such as TZDs and metformin.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

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