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RESEARCH ARTICLE

Acute stimulation of glucose influx upon mitoenergetic dysfunction requires LKB1, AMPK, Sirt2 and mTOR–RAPTOR

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ABSTRACT
Mitochondria play a central role in cellular energy production, and their dysfunction can trigger a compensatory increase in glycolytic flux to sustain cellular ATP levels. Here, we studied the mechanism of this homeostatic phenomenon in C2C12 myoblasts. Acute (30 min) mitoenergetic dysfunction induced by the mitochondrial inhibitors piericidin A and antimycin A stimulated Glut1-mediated glucose uptake without altering Glut1 (also known as SLC2A1) mRNA or plasma membrane levels. The serine/threonine kinase B1 (LKB1; also known as STK11) and AMP-activated protein kinase (AMPK) played a central role in this stimulation. In contrast, ataxiatelangiectasia mutated (ATM; a potential AMPK kinase) and hydroethidium (HEt)-oxidizing reactive oxygen species (ROS; increased in piericidin-A- and antimycin-A-treated cells) appeared not to be involved in the stimulation of glucose uptake. Treatment with mitochondrial inhibitors increased NAD⁺ and NADH levels (associated with a lower NAD⁺:NADH ratio) but did not affect the level of Glut1 acetylation. Stimulation of glucose uptake was greatly reduced by chemical inhibition of Sirt2 or mTOR–RAPTOR. We propose that mitochondrial dysfunction triggers LKB1-mediated AMPK activation, which stimulates Sirt2 phosphorylation, leading to activation of mTOR–RAPTOR and Glut1-mediated glucose uptake.

KEY WORDS: Glucose, Piericidin A, Antimycin A, NADH, Acetylation

INTRODUCTION
Glucose is one of the main substrates for ATP production and enters the cell through a family of facilitative glucose transporters (Gluts) (Zhao and Keating, 2007). Glut-mediated glucose transport depends on the difference between the extracellular ([GLC]e) and cytosolic glucose ([GLC]c) concentration. In the cytosol, glucose is phosphorylated by hexokinase to form glucose-6-phosphate (G6P). This process co-determines the magnitude of the glucose gradient across the plasma membrane and, thereby, the rate of Glut-mediated glucose uptake (Barros et al., 2007). Glucose is converted by the glycolysis pathway into pyruvate, which generates ATP. Pyruvate can be taken up by the mitochondria, where it is converted into acetyl coenzyme A. Subsequent oxidation through the tricarboxylic acid (TCA) cycle yields NADH and FADH₂, which are oxidized by complex I and complex II, respectively, of the mitochondrial oxidative phosphorylation (OXPHOS) system. The OXPHOS system uses the energy released through electron transport to generate ATP through chemiosmotic coupling (Mitchell, 1961). Each OXPHOS complex comprises multiple subunits and requires the assistance of proteinaceous assembly factors to catalyze its biogenesis (Agin-Perez and Enriquez, 2014; Pouwels et al., 2012). Mutations in structural subunits or assembly chaperones induce mitochondrial disease in humans (Koene et al., 2012; Loefen et al., 2000; van den Heuvel and Smeitink, 2001). Inherited complex-I deficiencies are the most common OXPHOS disorders (Bénit et al., 2009), whereas myopathy has been linked to an off-target effect of statins on complex III (Schirris et al., 2015). Moreover, empirical evidence suggests that inhibition of complex I mediates the action of the antidiabetic drug metformin and is relevant in cancer treatment (Chen et al., 2007; Schöckel et al., 2015; Vatrinet et al., 2015; Zhang et al., 2014). In addition to genetic errors of energy metabolism, mitoenergetic dysfunction has also been linked to diabetes, obesity and neurodegenerative disorders (Carelli et al., 2009; Chandra and Singh, 2011; Correa et al., 2012; Costa and Scorrano, 2012; Finsterer and Mahjoub, 2013; Koopman et al., 2013; Martin, 2011; Patti and Corvera, 2010; Rivie et al., 2010; Swerdlow, 2012).

To cope with mitoenergetic dysfunction and prevent energy crisis, cells can increase their glycolytic rate and thereby ATP production (Elstrom et al., 2004; Epstein et al., 2014; Liemburg-Apers et al., 2015a). For instance, an increased glycolytic rate has been observed in fibroblasts from individuals with myoclonic epilepsy and ragged-red fibers (MERRF) syndrome (Wu and Wei, 2012) and in cells chronically treated with the complex-I inhibitor rotenone (Distelmaier et al., 2015). Also, silencing of the complex-I gene NDUF5 triggers a glycolytic switch (Suhan et al., 2013). To increase the glycolytic rate, cells can stimulate Glut-mediated glucose uptake (Barros et al., 2007). The Glut family comprises 14 members, among which Glut1 (also known as SLC2A1) is the best-studied and most abundant isoform in proliferating cells (Mann et al., 2003). Glut1 is an integral membrane glycoprotein comprising 12 membrane-spanning α-helices (Mueckler et al., 1985). Mechanistically, Glut1 transport capacity can be stimulated by (i) increasing the translocation of Glut1-containing vesicles from the cytosol to the plasma membrane (Cura and Carruthers, 2012; Jing et al., 2008; Wheeler, 1988) or (ii) stimulating the activity of Glut1 transporters that are already present at the plasma membrane (Abbud et al., 2000; Barnes et al., 2002; Hamrahian et al., 1999; Loaiza et al., 2003; Mercado et al., 1989; Shetty et al., 1993; Shi et al., 1995). In a recent study (Liemburg-Apers et al., 2015a), we have demonstrated that acute (30 min) inhibition of complex I (with piericidin A) or of complex III (with antimycin A) increases glucose uptake and consumption in C2C12 myoblasts. This suggests that...
mitoenergetic dysfunction evokes a rapid compensatory increase in steady-state glucose uptake and consumption to prevent energy crisis in cells that are compromised in mitochondrial ATP generation. Here, we provide evidence suggesting that this compensatory process involves the stimulation of Glut1 activity through a mechanism requiring LKB1 (also known as STK11), AMPK, Sirt2 and the mTOR–Raptor complex.

RESULTS
Measurement of glucose uptake in C2C12 myoblasts during acute OXPHOS inhibition
To measure glucose levels, C2C12 myoblasts were transfected with the glucose sensor FLII-2Pglu-700μd6 (FLII) (Takanaga et al., 2008) and imaged using epifluorescence microscopy. FLII comprises a glucose-binding domain attached to CFP and the YFP-variant citrine. Glucose binding and unbinding induces conformational changes that affect the efficiency of fluorescence resonance energy transfer (FRET) from CFP to citrine. Previously, we have calibrated the citrine:FRET:CFP emission ratio signal in C2C12 cells, allowing quantification of the free cytosolic glucose concentration ([GLC]c) (Liemburg-Apers et al., 2015a). To study the effects of mitochondrial dysfunction, we used our recently described incubation protocol (Liemburg-Apers et al., 2015a). First, cells were pretreated (30 min) with vehicle (0.01% ethanol), the complex-I inhibitor piericidin A (100 nM), or the complex-III inhibitor antimycin A (20 nM). Mitochondrial inhibitors were used at concentrations that fully blocked mitochondrial O2 consumption (Liemburg-Apers et al., 2015a). Next, cytosolic glucose was depleted by placing the cells in a glucose-free medium for 18 min. During the last 15 min of this incubation, glucose consumption was blocked by adding the glycolaldehyde 3-phosphate dehydrogenase (GAPDH) inhibitor iodoacetic acid (IAA; 500 µM). Importantly, under these conditions glucose consumption was completely blocked (Liemburg-Apers et al., 2015a). This allowed quantification of the maximum rate of [GLC]c increase (Fig. 1A; lines), which is a measure of the maximal rate of glucose uptake, upon extracellular addition of 2 mM of glucose (Liemburg-Apers et al., 2015a).

Acute OXPHOS inhibition stimulates Glut1-mediated glucose uptake without increasing Glut1 levels at the plasma membrane
Piericidin A and antimycin A treatment increased the rate of glucose uptake (Fig. 1A,B). Quantitative QPCR analysis revealed that Glut1 was expressed, whereas mRNAs for Glut2, Glut3 and Glut4 (also known as SLC2A2, SLC2A3 and SLC2A4, respectively) were not detected (Fig. 1C). Glut1 mRNA levels were unaltered by inhibitor treatment (piericidin A and antimycin A), whereas exposure to CoCl2 (250 µM, 4.5 h), a treatment previously demonstrated to increase Glut1 expression (Barnes et al., 2002; Behrooz and Ismail-Beigi, 1997), increased these levels without inducing expression of the other Glut isoforms (Fig. 1C). In principle, piericidin A and antimycin A treatment could increase glucose uptake by stimulating the translocation of Glut1 from the cytosol to the plasma membrane (Barnes et al., 2002). Also, IAA treatment might affect Glut1 levels at the plasma membrane given the proposed involvement of GAPDH in vesicular transport (Zala et al., 2013). However, immunocytochemical analysis of Glut1 localization did not reveal obvious changes in cytosolic versus plasma membrane levels (Fig. 1D). Similarly, quantification of Glut1 levels at the plasma membrane using a biotinylation assay of cell surface Glut1 and cell-impermeable biotin (Barnes et al., 2002; Cura and Carruthers, 2012) revealed no alteration in the levels of biotinylated Glut1 (Fig. 1E). Taken together, these results suggest that the acute stimulation of glucose uptake by piericidin A and antimycin A is mediated by Glut1 but is not due to increased Glut1 levels at the plasma membrane.

Stimulation of Glut1-mediated glucose uptake by acute OXPHOS requires AMPK activation
During metabolic dysfunction, increased Glut1-mediated glucose uptake has been linked to phosphorylation of residue Thr172 (activation) of the energy stress sensor AMP-activated protein kinase (AMPK) (Abbud et al., 2000; Barnes et al., 2002; Cura and Carruthers, 2012; Hardie, 2015; Jing et al., 2008; Wu et al., 2013). Piericidin A and antimycin A treatment increased the levels of Thr172-phosphorylated AMPK (pAMPK; Fig. 2A). Also, the (inhibitory) phosphorylation of residue Ser79 of the pAMPK effector acetyl-CoA carboxylase (yielding pACC) appeared to be increased in piericidin-A- and antimycin-A-treated cells (Fig. 2B). The latter suggests that the increased level of pAMPK is paralleled by increased AMPK activity. Exogenous AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; 500 µM, 30 min) increased both pAMPK levels (Fig. 2C) and glucose uptake (Fig. 2D), although to a lesser extent than that with piericidin A. AICAR did not display an additive effect in combination with piericidin A, suggesting that AICAR and piericidin A stimulate glucose uptake through the same mechanism. To determine whether AMPK activity was required for piericidin-A- and antimycin-A-induced stimulation of glucose uptake, cells were pretreated with the cell-permeable AMPK inhibitor Compound C (20 µM, 2 h). This compound binds to the AMPK α2 catalytic subunit (encoded by PRKA42) in a pocket that partially overlaps with the putative ATP-binding site (Handa et al., 2011). Pretreatment with Compound C reduced the piericidin-A- and antimycin-A-stimulated increase in glucose uptake (Fig. 2E) and somewhat lowered antimycin-A-stimulated pACC formation (Fig. 2F). These findings suggest that AMPK activity is required for inhibitor-induced stimulation of Glut1-mediated glucose uptake. However, Compound C can also display off-target effects (Bain et al., 2007; Vogt et al., 2011). Therefore, we next used a knockdown strategy with small interfering (si)RNAs co-targeting the α1 (PRKAA1) and α2 catalytic subunits of AMPK (Cura and Carruthers, 2012). As intended, AMPK knockdown lowered the levels of pAMPK in vehicle-, piericidin-A- and antimycin-A-treated cells (Fig. 2G). This was paralleled by a reduction in inhibitor-stimulated glucose uptake (Fig. 2H). The above findings suggest that acute OXPHOS inhibition activates glucose uptake through a mechanism involving activation of AMPK through phosphorylation at Thr172.

Stimulation of Glut1-mediated glucose uptake by acute OXPHOS inhibition requires LKB1
In mammals, AMPK Thr172 phosphorylation is primarily mediated by two upstream kinases (Hardie et al., 2012): (i) a complex comprising LKB1, STRAD (STRADA) and MO25 (CAB39), which was originally identified as a tumor suppressor and (ii) Ca2+/calmodulin-activated protein kinase β (CaMKK1 or CaMKK2). Mechanistically, LKB1–STRAD–MO25 induces a high basal level of Thr172 phosphorylation that is modulated by the binding of AMP to the AMPK γ-subunit (Xie et al., 2006). The latter stimulates AMPK phosphorylation and inhibits its dephosphorylation (Davies et al., 1995; Hawley et al., 1995). LKB1 is activated by phosphorylation of Ser431 (yielding pLKB1) and a variety of conditions (Gan and Li, 2014). Piericidin A and antimycin A
treatment apparently increased the level of pLKB1 (Fig. 3A). Knockdown of LKB1 reduced the inhibitor-induced stimulation of AMPK Thr172 phosphorylation (Fig. 3B). Surprisingly, LKB1 knockdown did not affect glucose uptake in vehicle- or piericidin-A-treated cells but reduced this parameter in antimycin-A-treated cells (Fig. 3C). These results suggest that although LKB1 is involved in mediating the stimulation of glucose uptake in antimycin-A-inhibited cells, additional modes of regulation are involved in piericidin-A-induced stimulation of glucose uptake. In this sense, the ataxia telangiectasia mutated (ATM) protein has been demonstrated to activate AMPK through an LKB1-independent mechanism in AICAR-treated cells (Sun et al., 2007). However, pretreatment with the ATM inhibitor KU55933 (1 μM, 1 h) (Hickson et al., 2004) did not significantly reduce the piericidin-A-induced increase in glucose uptake (Fig. 3D), suggesting that ATM is not a primary regulator of glucose uptake in our model.

Stimulation of Glut1-mediated glucose uptake by acute OXPHOS inhibition is not prevented by antioxidants

Both inhibitor-induced and inherited mitochondrial dysfunction are generally characterized by increased ROS levels (Forkink et al., 2015a; Koopman et al., 2013, 2012; Willems et al., 2015; Zorov et al., 2014). These ROS might be involved in the regulation of cellular glucose metabolism (Liemburg-Apers et al., 2015b). Although still controversial, it has been proposed that ROS and/or changes in redox state might also activate AMPK (Han et al., 2010; Jensen et al., 2008; Mungai et al., 2011; Wu and Wei, 2012; Zmijewski et al., 2010). Using a previously described strategy (Forkink et al., 2015b; Grefte et al., 2015), we quantified the oxidation of the ROS sensor hydroethidium (HEt; Fig. 4A). This revealed that antimycin A and piericidin A increased the levels of HEt-oxidizing ROS (Fig. 4B). Increased piericidin-A-induced HEt oxidation was unaffected by pretreatment with the antioxidant ...
that the pAMPK level and glucose uptake displayed a sigmoidal relationship (Fig. 4E) and pAMPK levels (Fig. 4F). Taken together, these results suggest that the elevated levels of HET-oxidizing ROS in piericidin-A-treated cells are not responsible for stimulation of the pAMPK-mediated increase in glucose uptake in cells with inhibited OXPHOS.

**Stimulation of Glut1-mediated glucose uptake by acute OXPHOS inhibition has a maximum level**

By combining data obtained in acutely treated cells, it was found that the pAMPK level and glucose uptake displayed a sigmoidal relationship (Fig. 5). This supports our above result that pAMPK is required for acute stimulation of Glut1-mediated glucose uptake and suggests that the latter has a limited capacity.

**Stimulation of Glut1-mediated glucose uptake by acute OXPHOS inhibition requires Sirt2 activity**

Chemical inhibition of complex I and complex III is associated with increased mitochondrial NADH levels (Forkink et al., 2015a). Similarly, genetic complex-I deficiency in mouse embryonic fibroblasts from *NDUFS4*−/− mice is characterized by lower cellular levels of NAD⁺ and increased levels of NADH (leading to a reduced NAD⁺:NADH ratio), without alterations in NAD⁺ and NADPH levels (Valsecchi et al., 2012). Using a heart-specific *NDUFS4*−/− mouse model, further evidence has been provided that complex-I deficiency is associated with a reduced NAD⁺:NADH ratio.
uptake in vehicle- and piericidin-A-treated cells. Statistics: mean±s.e.m. represents three independent experiments (A–D, >50 cells were analyzed per condition. Significant differences with the indicated columns and conditions (indicated by letters) are as marked; *P<0.05, **P<0.01 and ***P<0.001; Dunn’s post-hoc test (A–D) and Bonferroni’s post-hoc test (B–D).

ratio and that this reduction is paralleled by a lower activity of the NAD+-dependent deacetylase sirtuin 3 (Sirt3) (Karamanlidis et al., 2013). As a consequence, protein acetylation is increased and heart failure accelerated. Here, we observed that piericidin A and antimycin A treatment increased the total cellular level of NADH (Fig. 6A; open bars), and possibly of NAD+ (Fig. 6B; open bars), resulting in a lower NAD+/NADH ratio (Fig. 6C; open bars). In AMPK-knockdown cells, the inhibitor-induced increase in NADH was fully prevented and NAD+ levels were decreased (Fig. 6A–C; filled bars). In order to assess a possible role for sirtuin proteins in our model system, we specifically inhibited sirtuin 2 (i.e. Sirt2), which displays a cytosolic localization (Poulose and Raju, 2015). In vehicle-treated cells, inhibition of Sirt2 activity by using the specific inhibitor AGK2 (5 μM, 2 h) (Outeiro et al., 2007) stimulated glucose uptake (Fig. 6D). This suggests that Sirt2 activity inhibits glucose uptake under this condition. Enigmatically, in piericidin-A- and antimycin-A-treated cells, increased glucose uptake was reduced upon Sirt2 inhibition, suggesting that Sirt2 activity is required to allow stimulation of glucose uptake (Fig. 6D). AGK2 treatment did not affect the inhibitor-induced increase in pAMPK levels, compatible with Sirt2 being a downstream effector of pAMPK (Fig. 6E). Next, it was assessed whether Sirt2 might stimulate glucose uptake through deacetylation of Glut1. First, all proteins with acetylated lysine residues were immunoprecipitated, and Glut1 levels were analyzed by western blotting. Indeed, Glut1 was detected, but its acetylation status was not significantly affected by piericidin A or antimycin A treatment (Fig. 6F). To confirm Glut1 acetylation, Glut1 was immunoprecipitated and acetyl-lysine was analyzed on a western blot. A band specific for acetyl-lysine was found to run at the same place on a gel as that for Glut1 (Fig. 6G). Again, the acetylation status of Glut1 was unaffected by piericidin A or antimycin A treatment (Fig. 6G). The above results suggest that Sirt2 is involved in piericidin-A- and antimycin-A-induced stimulation of glucose uptake, but that Glut1 is not a direct deacetylation target of Sirt2.

**Stimulation of Glut1-mediated glucose uptake by acute OXPHOS inhibition requires the activity of mTOR–RAPTOR**

A previous study has suggested that AMPK can phosphorylate Sirt2 at Thr101 and that this phosphorylation is required for the interaction with the serine/threonine protein kinase Akt (of which there are three isoforms) (Ramakrishnan et al., 2014). This interaction between Sirt2 and Akt is required to allow optimal Akt activation. In this sense, previous evidence has revealed that Akt inhibitors effectively block the mammalian target of rapamycin complex 1 (mTORC1; containing mTOR and RAPTOR) and decrease glucose uptake, glycolytic rate and cell viability (Rashmi et al., 2014). In agreement with our above results, it has been concluded previously that mTOR–RAPTOR promotes Glut1 activity but does not regulate Glut1 surface localization (Wieman...
et al., 2007). In order to assess the potential involvement of Akt in the piericidin-A- and antimycin-A-induced increase in glucose uptake, we tested the pan-Akt inhibitor SC-66 (Jo et al., 2011). Pretreatment with SC-66 (10 μg/ml, 1 h) (Rashmi et al., 2014) did not reduce the stimulation of glucose uptake in inhibitor-treated cells (data not shown). Assuming that the effect of SC-66 is Akt specific, this result argues against involvement of Akt in the stimulation of glucose uptake. Next, to assess the potential involvement of mTOR-RAF in the piericidin-A- and antimycin-A-induced increase in glucose uptake, we used the mTOR inhibitor rapamycin. Pretreatment with rapamycin (25 nM, 24 h) (Wieman et al., 2007) greatly reduced the stimulation of glucose uptake in inhibitor-treated cells (Fig. 6H), supporting the hypothesis that mTOR-RAF promotes Glut1 transporter activity (Wieman et al., 2007). The above results suggest that mTOR-RAF is involved in piericidin-A- and antimycin-A-induced stimulation of glucose uptake, potentially through an Akt-independent mechanism.

DISCUSSION

Increased Glut1-mediated glucose uptake during metabolic stress is an important cellular adaptation mechanism. Understanding the latter is of relevance for a broad spectrum of metabolic disorders, including mitochondrial dysfunction, cancer and diabetes. Here, we show that 30 min of inhibition of mitochondrial complex I or complex III increases the rate of glucose uptake without increasing the abundance of Glut1 at the plasma membrane. This suggests that Glut1 preexisting at the plasma membrane becomes activated. Our findings suggest that Glut1 activation is mediated by a signaling cascade involving LKB1, AMPK, Sirt2 and mTOR-RAF.

Although stimulation of glucose uptake through short-term inhibition of OXPHOS complexes has been described previously,
its underlying mechanism still remains poorly understood (Barros et al., 1995; Hamrahian et al., 1999; Jing et al., 2008; Koseoglu and Beigi, 1999; Liemburg-Apers et al., 2015a; Mercado et al., 1989; Shetty et al., 1993; Shi et al., 1995). Here, we provide evidence that mitochondrial inhibition neither increases Glut1 mRNA levels nor Glut1 abundance at the plasma membrane, which is compatible with the relatively fast stimulation of glucose uptake. Inhibition of ATM, a protein that promotes Glut1 plasma membrane localization in myoblasts (Andrisse et al., 2013), did not diminish the increased rate of glucose uptake induced by piericidin A. This suggests that Glut1 internalization, as well as the translocation of Glut1-containing vesicles to the plasma membrane, is unaffected by acute mitochondrial inhibition. In other cell types, metabolic stress increases the activity of Glut1 that is already localized at the plasma membrane (Abbud et al., 2000; Barnes et al., 2002; Koseoglu and Beigi, 1999; Shetty et al., 1993; Shi et al., 1995). Our previous results have demonstrated that piericidin A and antimycin A increase the $V_{\text{max}}$ of the Glut1-mediated glucose uptake without affecting the affinity ($K_c$) of this process (Liemburg-Apers et al., 2015a). Taken together, the above data suggest that inactive Glut1 molecules at the plasma membrane are activated during mitochondrial inhibition. For instance, ATP allosterically inhibits Glut1 and therefore $V_{\text{max}}$ is increased when ATP levels are reduced (Carruthers and Helgerson, 1989). The latter might occur upon acute mitochondrial inhibition and trigger a compensatory increase in glucose uptake. However, Glut1 activity remains increased after ATP levels return to normal in clone 9 cells with an inhibited complex I (Mercado et al., 1989). This is compatible with the normal total cellular ATP levels measured after 24 h in piericidin-A- and antimycin-A-treated C2C12 cells (Liemburg-Apers et al., 2015a).

We observed that piericidin A and antimycin A treatment triggered AMPK activation. In addition, AICAR-induced AMPK activation was paralleled by an increase in glucose uptake under vehicle-treated conditions. AICAR did not further increase glucose uptake in piericidin-A-treated cells, suggesting that AICAR and piericidin A stimulate this uptake through the same mechanism. The latter observation is compatible with previous studies showing that short-term metabolic stress stimulates Glut1-mediated glucose uptake in an AMPK-dependent manner (Abbud et al., 2000; Barnes et al., 2002; Jing and Ismail-Beigi, 2007). Importantly, acute chemical inhibition of AMPK activity or knockdown of its $\alpha_1$ and $\alpha_2$ subunits partially inhibited the increased glucose uptake that was induced by mitochondrial inhibition. This partial inhibition might be due to the fact that AMPK phosphorylation was still higher in piericidin-A or antimycin-A-treated than in vehicle-treated cells. Piericidin A and antimycin A treatment slightly increased the levels of pLKB1; this is compatible with a mechanism in which a high basal LKB1 activity continuously stimulates Thr172 phosphorylation on AMPK, which is immediately counterbalanced by dephosphorylation when AMP is low (Hardie, 2011). Acute mitochondrial inhibition increases the AMP:ATP ratio (Jing and Ismail-Beigi, 2007; Mercado et al., 1989; Shetty et al., 1993), and AMP binding to the AMPK $\gamma$-subunit enhances phosphorylation of the $\alpha$-subunit by the upstream AMPK kinase LKB1 (Hardie and Ashford, 2014; Shaw et al., 2004; Woods et al., 2003). In addition, AMP binding induces a conformational change that inhibits AMPK dephosphorylation (Davies et al., 1995; Sanders et al., 2007). LKB1 knockout significantly inhibited piericidin-A- and antimycin-A-induced AMPK activation but only prevented stimulation of glucose uptake in antimycin-A-treated cells. This might suggest that piericidin A activates AMPK through an alternative route when LKB1 levels are low. Piericidin A treatment increased ROS levels to a greater extent than antimycin A treatment. It has also been suggested that increased ROS levels and/or ATM activation can also (in)directly activate AMPK (Guo et al., 2010; Kurz et al., 2004; Zmijewski et al., 2010). However, treatment with antioxidants and ATM inhibition were unable to prevent the increase in glucose uptake upon piericidin-A treatment. Of note, the antioxidants Trolox and Tempol only partially diminished piericidin-A-induced HET oxidation, leaving open the possibility that the remaining ROS increase might still suffice to activate AMPK and/or Glut1. Alternatively, AMPK-independent signaling might be involved, although this is less likely given the fact that AMPK knockdown completely prevented the piericidin-A-induced stimulation of glucose uptake.

The mechanism by which AMPK stimulates Glut1-mediated glucose uptake under conditions of metabolic stress has been previously studied (Abbud et al., 2000; Barnes et al., 2002; Jing et al., 2008). Co-immunoprecipitation studies reveal that pAMPK does not bind to Glut1 under metabolic stress conditions (Jing and Ismail-Beigi, 2007), suggesting that Glut1 is not directly phosphorylated by AMPK. We observed that NADH levels were increased in piericidin-A- and antimycin-A-treated cells, which is probably due to the combined effects of decreased OXPHOS-mediated NADH oxidation and increased glycolysis-mediated NADH production. However, NAD$^+$ levels were not reduced and even appeared increased in inhibitor-treated cells. This might suggest that acute mitochondrial inhibition stimulates de novo synthesis of NAD$. Interestingly, the inhibitor-induced increase in NADH and NAD$^+$ was prevented by AMPK knockdown. Mitochondrial dysfunction and changes in AMPK activity have previously been linked to alterations in NADH and NAD$^+$ levels, the latter of which acts as a co-factor for sirtuin proteins (Forkink et al., 2015a; Karamanlidis et al., 2013). Sirtuins are protein deacetylases involved in cell cycle regulation, genomic stability and energy metabolism (Choudhary et al., 2009, 2014). In contrast...
However, we observed that Sirt2 inhibition did not diminish leading to AMPK activation (Hou et al., 2008; Lan et al., 2008). In the nucleus, Sirt1 deacetylates LKB1, a n and / or is not a target of Sirt2. suggesting that Sirt2 operates downstream of AMPK. A large number of glycolytic enzymes can be acetylated, and reversible acetylation is believed to fine-tune metabolic adaptation and insulin signaling in muscle (LaBarge et al., 2015; Philp et al., 2014). We here observed that the Glut1 acetylation status was similar under basal and OXPHOS-inhibited conditions, suggesting that Glut1 is not directly modulated by acetylation and/or is not a direct target of Sirt2.

A potential downstream target of Sirt2 is Akt, which is involved in insulin signaling and promotes a glycolytic phenotype in various cell types (Elstrom et al., 2004; Higaki et al., 2008; Ramakrishnan et al., 2014). Acetylation of Akt at residue Lys20 prevents plasma membrane localization and activation of Akt by

to other sirtuins, Sirt2 localizes predominantly in the cytosol (North et al., 2003; Rauh et al., 2013). This suggests that Sirt2 might link AMPK phosphorylation to Glut1 activation. Supporting this hypothesis, Sirt2 inhibition partially prevented the piericidin-A- and antimycin-A-induced increase in glucose uptake. However, we also observed that Sirt2 inhibition stimulated glucose uptake under the vehicle-treated condition. This suggests that Sirt2 might prevent excessive glucose uptake under normal conditions, whereas it mediates stimulation of glucose uptake during metabolic stress. In the nucleus, Sirt1 deacetylates LKB1, leading to AMPK activation (Hou et al., 2008; Lan et al., 2008). However, we observed that Sirt2 inhibition did not diminish piericidin-A- and antimycin-A-induced AMPK phosphorylation,
phosphatidylinositol 3-kinase (PI3K) (Sundaresan et al., 2011), whereas deacetylation of Akt by Sirt2 is reported to promote its activity (Chen et al., 2013; Dan et al., 2012). It has been previously suggested that phosphorylation of Thr101 on Sirt2 by AMPK is required for an Akt–Sirt2 interaction and subsequent activation by PI3K upon insulin signaling (Ramakrishnan et al., 2014). However, using the Akt inhibitor SC-66, we were unable to demonstrate the above Akt involvement in our model system. In contrast, inhibition of mTOR–RAPTOR greatly reduced the piericidin-A- and antimycin-A-induced stimulation of glucose uptake. This is compatible with the reduced glycolytic flux observed in rapamycin-treated NDUFS4−/− mice with an isolated complex-I deficiency (Johnson et al., 2013).

Taken together, our results suggest that the activity of LKB1, AMPK, Sirt2 and mTOR–RAPTOR is required for the stimulation of cellular glucose uptake induced by mitochondrial dysfunction. We propose a mechanism (Fig. 7) in which acute inhibition of complex I or complex III (transiently) increases AMP levels and decreases ATP levels. These changes inhibit AMPK dephosphorylation and stimulate LKB1-dependent AMPK activation. Activated AMPK phosphorylates Sirt2, which leads to activation of mTOR–RAPTOR and of Glut1 through an unknown but possible Akt-independent mechanism.

**MATERIALS AND METHODS**

**Chemicals**

Sodium iodoacetate (IAA), antimycin A, AICAR, 2-cyano-3-[5-(2,5-dichlorophenyl)-2-furanyl]-N-5-quinolinyl-2-propenamide (AGK2), trichostatin A (TSA) and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4-hydroxy-TEMPO or ‘Tempol’) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Piericidin A was obtained from Enzo Life Sciences (Raamsdonksveer, The Netherlands). 6-[4-[[2-(1-Piperidinyl)ethoxy]-N-5-quinolinyl]-3-(4-pyridinyl)pyrazolo[1,5-a]pyrimidine (Dorsomorphin or Compound C) was obtained from Calbiochem (Billericia, MA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka (Buchs, Switzerland). 2-morpholino-6-(thianthren-1-yl)-4H-pyran-4-one (KU55933) was obtained from Abcam (Cambridge, UK). Rapamycin was obtained from LC Laboratories (Woburn, MA).

**Cell culture**

C2C12 myoblasts were obtained from American Type Culture Collection (Wesel, Germany) and cultured at 37°C (95% air, 5% CO2) in Dulbecco’s modified eagle’s medium (DMEM-32430; Life Technologies Invitrogen, Bleiswijk, The Netherlands), supplemented with 10% (v/v) fetal bovine serum (FBS) (10270-106; Life Technologies Invitrogen). For glucose measurements, myoblasts were seeded onto Fluorodishes® (#FD35-100; World Precision Instruments, Sarasota, FL) at a density of 40,000 cells/dish.

**Transfection**

One day after seeding, C2C12 cells were at 40% confluence. DNA (1 µg) was mixed with 6 µl of Lipofectamine (Life Technologies Invitrogen) in 0.5 ml FBS-free DMEM. Following a 20-min incubation at room temperature, the transfection mix was added to the cells in FBS-free DMEM. After 6 h, this medium was replaced by DMEM containing 10% FBS. The glucose sensor FLIIδPglu-700µδδ (FLII) (Addgene Plasmid #17866) (Takanaga et al., 2008) was created in the laboratory of Dr Wolf Frommer (Department of Plant Biology, Carnegie Institution for Science, CA).

**Single-cell glucose uptake measurements**

These experiments were performed as described in detail previously (Liemburg-Apers et al., 2015a). Briefly, cells were washed and incubated for 3 min in HEPES-Tris buffer (adjusted to pH 7.4 with Tris-HCl) containing 4.2 mM KCl, 132 mM NaCl, 10 mM HEPES, 1.2 mM MgCl2 and 1 mM CaCl2. Next, cells were placed on an inverted microscope (Axiovert 200 M, Carl Zeiss BV, Sliedrecht, The Netherlands). FLII was excited at 430 nm (CFP) for 300 ms, CFP fluorescence was detected using a 455DRLP dichroic mirror (Omega Optical, Brattleboro, VT) and a 480AF30 emission filter (Omega). Citrine emission (citrineFLII) was
measured using a 430-nm excitation light (for 200 ms), and emission was detected using the 455DRLP dichroic mirror and a 535AF26 emission filter (Omega). Time-lapse recordings were performed using an acquisition interval of 2.5 s. Microscopy images were stored in native MetaFluor format (Universal Imaging Corporation, Downingtown, PA) and analyzed off-line using Image Pro Plus 6.1 software (Media Cybernetics, Rockville). Individual images were background-corrected for the individual wavelengths by subtracting the average intensity in an extracellular region of interest. Next, cell-derived fluorescence signals were quantified using cytosolic circular regions of interest. CITrinesFRET:CFP emission ratios were converted to [GLC], using a previously generated in situ calibration curve (Liemburg-Apers et al., 2015a).

**QPCR analysis**
This was performed as described previously (Liemburg-Apers et al., 2015a). The primer sequences (forward and reverse) were: Glut1, 5′-TGCAGTT-CGGCCTATAACCTG-3′ and 5′-GGTGGTCCATGGTTGATG-3′; Glut2, 5′-TGTGATCAATGCACCTCAC-3′ and 5′-TCAATGTT-AATGGCAGCTTTCC-3′; Glut3, 5′-ACTCTTGGTCAACGGCTTTG-3′ and 5′-ACTCTTGCCGAACTCATAAAG-3′; Glut4, 5′-CTAAAGCCACCTGAAA-3′ and 5′-TACGCCAGAGGCTAC-CAGG-3′.

**Preparation of cell lysates**
Cells were scraped and centrifuged at 600 g for 5 min at 4°C. Cell pellets were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 1% (w/v) deoxycholate, 1% (w/v) Triton X-100, 10 mM Na₂PO₄, 50 mM NaF, 0.1 mg/ml DNase and 1 μg/ml protease inhibitor cocktail. After 30 min of incubation under gentle rotation at 4°C, cell suspensions were centrifuged at 15,000 g for 10 min at 4°C, and supernatants were used for protein concentration determination by measuring the absorption at 280 nm using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

**Western blot analysis**
Proteins were denatured (70°C; 10 min) in sample buffer containing 250 mM Tris-HCl (pH 6.8), 160 mM dithiothreitol, 2% (v/v) SDS, 12% (v/v) glycerol and 0.03% (w/v) Bromophenol Blue. Proteins (30 μg/lane) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes (Novex, Thermo Fisher Scientific). Membranes were blocked with Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) diluted 1:1 in PBS (v/v) and incubated with primary antibodies. Polyclonal anti-Glut1 (1:500; #07-1401), monoclonal anti-LKB1 (1:1000; #05-832) and polyclonal anti-pLKB1 (Ser431) (1:1000; #09-495) antibodies were obtained from Millipore (Billerica, MA). Polyclonal anti-AMPK (1:1000; #2532L), monoclonal anti-pAMPK (Thr172) (1:1000; #2535L) and anti-p-AKT (Ser473) (1:1000; #4060S) antibodies were obtained from Cell Signaling (Bioké, Leiden, The Netherlands). Monoclonal anti-p-AKT (Ser473) (1:1000; #4060S) antibody was obtained from Sigma. For detection, IRDye 680RD-conjugated goat anti-mouse IgG (1:10,000) and IRDye 800CW-conjugated goat anti-rabbit IgG (1:10,000) (Li-Cor, Lincoln, NE) secondary antibodies were used. Membranes were scanned, and band intensities were quantified with Image Studio Lite software (Li-Cor; version 4.0).

**Immunocytochemistry of Glut1**
After treatments, cells were fixed using 4% (v/v) formaldehyde in PBS for 15 min. Immunocytochemistry was performed as described in detail previously (Greffe et al., 2015). Briefly, cells were permeabilized in 0.5% (v/v) Triton X-100 in PBS for 20 min. After washing, cells were blocked in blocking buffer containing 2% (w/v) bovine serum albumin (BSA), 2% (v/v) normal goat serum (NGS), 0.1% (v/v) Triton X-100, 0.05% (v/v) Tween-20 and 100 mM glycine in PBS for 30 min. Cells were incubated with mouse anti-Glut1 (1:100; 07-1404, Millipore) antibody in blocking buffer without glycine for 1 h. Glut1 was detected with Alexa-Fluor-488-labeled goat anti-rabbit antibody (1:200; Molecular Probes, Leiden, The Netherlands). Cells were sealed using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI), which stains all nuclei (Vector Laboratories, Burlingame, CA). Images were taken on an Olympus FV1000 confocal laser-scanning microscope.

**Cell surface protein biotinylation**
Biotinylation was performed using the Pierce cell surface protein isolation kit (89881; Thermo Fisher Scientific) according to the manufacturer’s protocol. For each condition, two confluent T175 culture flasks were used. After treatment, cells were incubated with Sulfo-NHS-SS-biotin, and cell membrane integrity was assessed visually and proven to be intact. Biotinylated proteins were purified using NeutrAvidin agarose resin and loaded onto a gel and analyzed by western blotting with an anti-Glut1 antibody (see above).

**Gene knockdown**
AMPK knockdown was performed by transfecting 75,000 cells with 60 ng of siRNA directed against AMPK α1 and AMPK α2 subunits (sc-45313) or scramble siRNA (sc-37007) (Santa Cruz Biocounter Life Sciences, Huissen, The Netherlands). LKB1 knockdown was performed by transfecting 75,000 cells with 100 nM siRNA directed against LKB1 (s74499) or a scrambled siRNA (Silencer select negative control No. 1) (Life Technologies Invitrogen). Transfection with siRNA was performed using the Neon Transfection System (Life Technologies Invitrogen) according to manufacturer’s protocol for C2C12 myoblasts. One day after siRNA addition, cells were transfected with FLII using Lipofectamine (see the above section on transfection). Two days after siRNA addition, cells were used for experiments.

**NAD⁺ and NADH measurements**
Per sample, 50,000 cells were seeded in a 6-well plate and used the next day for measurements. For AMPK-knockdown experiments, cells were transfected with siRNA, seeded in 6-well plates and used for measurement 2 days later. Cells were treated for 30 min with mitochondrial inhibitors, after which they were scraped, and NAD⁺ and NADH were quantified using the EnzyFluo™ NAD⁺:NADH assay kit (EFND-100, BioAssay Systems, Hayward, CA) according to the manufacturer’s protocol. Upon the addition of reagent, the optical density (OD) at 560 nm was measured using a 2-min interval on the Synergy 2 multi-mode reader (Biotek, Abcoude, The Netherlands). The ΔOD (10 min) values were normalized to those for the vehicle-treated condition for each experiment.

**HEt oxidation**
The levels of HEt-oxidizing ROS were determined as described in detail previously (Forkink et al., 2015; Greffe et al., 2015). Cells were pretreated for 30 min with mitochondrial inhibitors followed by an 18-min incubation in the absence of glucose, of which the last 15 min were in the presence of IAA and the last 5 min in the presence of 10 μM HEt (Life Technologies Invitrogen). Next, the cells were washed with HEPES-Tris buffer, and Fluorodishes® were mounted in an incubation chamber and placed on the stage of an inverted microscope (Carl Zeiss, see above). Cells were excited at 490 nm for 100 ms, and fluorescence emission was detected by a 565ALP emission filter (Omega). Quantitative analyses were performed using Metamorph 6.0 software (Universal Imaging Corporation). The mean fluorescence intensity in a mitochondria-dense and nuclear region was determined for at least ten different microscopic fields and corrected for background intensity using an extracellular region of identical size. For each experiment, two dishes and a minimum of 30 cells were analyzed per tested condition.
PBS, and proteins were eluted in 30 µl of 4x sample buffer at 70°C for 10 min and analyzed by western blotting. Acetylated Glut1 was quantified by normalizing the acetylation signal to the Glut1 signal.

Statistical analysis
Average values are presented as mean±s.e.m. Curve fitting and statistical analysis were performed using Origin Pro 6.1 (OriginLab Corp., Northampton, USA) and GraphPad Prism 5 (Graphpad Software, La Jolla, CA). Unless stated otherwise, statistical significance relative to the indicated condition was determined using a Kruskal–Wallis test (followed by a Dunn’s post-hoc test) or a two-way ANOVA (followed by Bonferroni post-hoc test); ***P<0.001, **P<0.01 and *P<0.05.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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