Cell Metabolism
Short Article

Interdependence of AMPK and SIRT1 for Metabolic Adaptation to Fasting and Exercise in Skeletal Muscle

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SUMMARY

During fasting and after exercise, skeletal muscle efficiently switches from carbohydrate to lipid as the main energy source to preserve glycogen stores and blood glucose levels for glucose-dependent tissues. Skeletal muscle cells sense this limitation in glucose availability and transform this information into transcriptional and metabolic adaptations. Here we demonstrate that AMPK acts as the prime initial sensor that translates this information into SIRT1-dependent deacetylation of the transcriptional regulators PGC-1α and FOXO1, culminating in the transcriptional modulation of mitochondrial and lipid utilization genes. Deficient AMPK activity compromises SIRT1-dependent responses to exercise and fasting, resulting in impaired PGC-1α deacetylation and blunted induction of mitochondrial gene expression. Thus, we conclude that AMPK acts as the primordial trigger for fasting- and exercise-induced adaptations in skeletal muscle and that activation of SIRT1 and its downstream signaling pathways are improperly triggered in AMPK-deficient states.

INTRODUCTION

During fasting or energy deficits, the limiting glucose availability must be compensated by an increase in mitochondrial fatty acid oxidation in skeletal muscle and other peripheral tissues to preserve blood glucose levels and supply glucose-dependent tissues, such as the brain or red blood cells (Cahill et al., 1966). The lack of metabolic flexibility to correctly adapt to energy demands and nutrient availability constitutes a burden on energy homeostasis, ultimately leading to metabolic disease (Kelley and Mandarino, 2000). Metabolic adaptations in muscle are driven by coordinated transcriptional responses to promote the mitochondrial use of lipid substrates as a source of energy (de Lange et al., 2006). Such alterations in gene expression patterns are achieved through the modulation of transcriptional regulators, such as the coactivator PGC-1α or the FOXO family of transcription factors, both of which are intimately linked to the regulation of mitochondrial and fatty acid metabolism (Gross et al., 2008; Handschin and Spiegelman, 2006). The activities of PGC-1α (Rodgers et al., 2005) and FOXOs (Brunet et al., 2004) are critically influenced by the control of their acetylation levels through the type III NAD^+-dependent deacetylase SIRT1. As a result, SIRT1 acts as master regulator of muscle adaptations to nutrient availability (Fulco et al., 2008; Gerhart-Hines et al., 2007). Notably, SIRT1 knockdown prevents the induction of mitochondrial and lipid oxidation genes in glucose-restricted myotubes (Gerhart-Hines et al., 2007). How SIRT1 activity is regulated is still largely unknown, even thought it has been hypothesized that NAD^+ availability, for which SIRT1 acts as a sensor, plays a major role (Gerhart-Hines et al., 2007).

Another metabolic sensor with strong impact on transcriptional responses is the AMP-activated protein kinase (AMPK), a heterotrimeric Ser/Thr kinase composed of one catalytic (α) and two noncatalytic subunits (β and γ) (Hardie, 2007). Two different isoforms exist for both the α and the β subunits (α1 and α2 or β1 and β2), while the γ subunits are encoded by three different genes (γ1, γ2, and γ3) (Hardie, 2007). The γ subunits can bind AMP or ATP in a competitive and mutually exclusive manner (Hardie, 2007). The binding of AMP increases the catalytic activity of the complex and enhances the phosphorylation of a key residue within the catalytic domain (Thr^{172}), essential for activity. In contrast, the binding of ATP antagonizes the effects of AMP on enzymatic activity, transforming AMPK into an energy-monitoring system of AMP:ATP ratio. The activation of AMPK turns on catabolic pathways to generate ATP, and switches off a number of processes that consume ATP, such as fatty acid, protein, or cholesterol synthesis (Hardie, 2007). Interestingly, the activity of AMPK seems connected to that of SIRT1. AMPK enhances SIRT1 activity by increasing intracellular NAD^+ levels (Canto et al., 2009; Costford et al., 2009; Fulco et al., 2008). This translates in the deacetylation of SIRT1 targets, such as PGC-1α, in response to pharmacological or physiological AMPK activation (Canto et al., 2009). Whether AMPK is required for fasting-induced activation of SIRT1 and deacetylation of its targets remains unknown. Here we provide evidence that AMPK acts as the primordial sensor of energy stress and that its activity is required to trigger SIRT1-dependent adaptations.

RESULTS

C2C12 myotubes display a number of adaptive changes after 48 hr incubation in glucose-restricted medium (5 mM). For

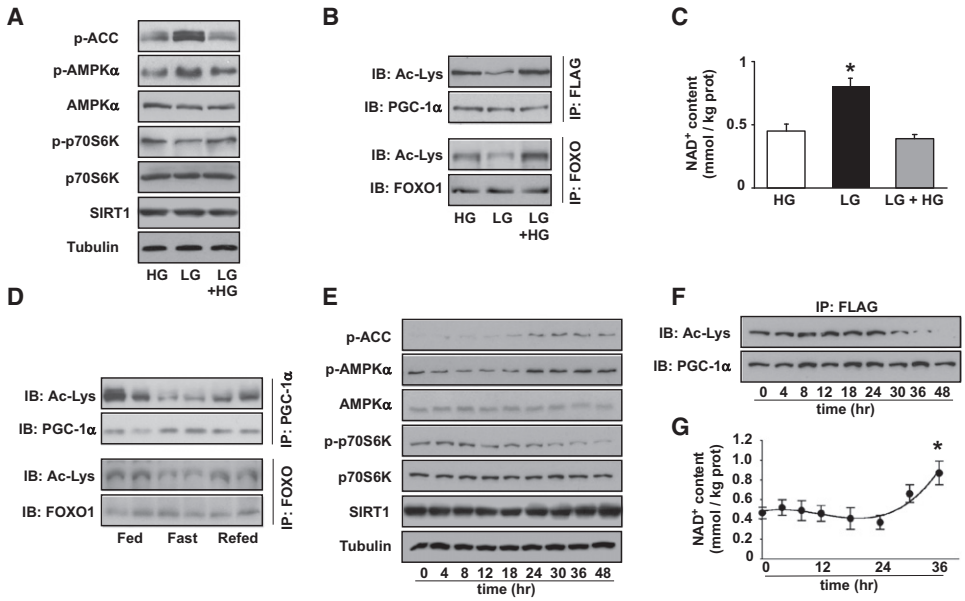

Figure 1. Glucose Restriction Increases PGC-1α-Dependent Gene Expression

(A–C) C2C12 myotubes were infected with adenoviruses encoding FLAG-HA-PGC-1α. Then, cells were left for 48 hr in medium containing either 25 mM glucose (HG) or 5 mM glucose (LG). A third group was treated by adding 25 mM glucose medium for 6 hr after 48 hr in LG (LG + 6 hr HG). (A) Total protein was obtained and 100 μg were used for western blot analysis to test the markers indicated. (B) Of total protein extracts, 500 μg was used for immunoprecipitation (IP) against FLAG antibody or against FOXO to determine the acetylation levels of PGC-1α or FOXO1, respectively. (C) Acidic lysates were obtained for measurement of NAD^+ levels. Data are presented as mean ± SE from 6 different experiments. * indicates statistical difference versus HG group.

(D) Nuclear extracts (300 μg) from gastrocnemius muscle from fed, fasted, or refed C57BL/6J mice were used for IP against PGC-1α or FOXO1 to measure acetylation.

(E–G) After infection with adenoviruses encoding FLAG-HA-PGC-1α, C2C12 myotubes were left in 5 mM glucose medium and samples were obtained at the times indicated. (E) Of total protein extracts, 100 μg was used for western analysis to test the markers indicated. (F) Of total protein extracts, 500 μg was used for immunoprecipitation to detect the acetylation levels of PGC-1α. (G) Acidic lysates were obtained for measurement of NAD^+ levels. Data are presented as mean ± SE from five different experiments. * indicates statistical difference versus t = 0. All images are representative of three to six independent experiments.

example, AMPK is activated, as manifested by the phosphorylation on the Thr^{172} residue of the α subunit and the phosphorylation of ACC, an AMPK substrate (Figure 1A). This was in parallel to the downregulation of the mTOR pathway, as evidenced by p70S6K phosphorylation (Figure 1A), and the deacetylation of SIRT1 targets, PGC-1α and FOXO1 (Figure 1B). The deacetylation of SIRT1 targets was not due to higher SIRT1 protein levels (Figure 1A) but rather due to changes in activity derived from increased NAD^+ levels (Figure 1C), which reflected a metabolic rearrangement toward oxidative metabolism. Consequent to the changes in PGC-1α and FOXO activity through deacetylation, the expression of mitochondrial and lipid oxidation genes was increased after 48 hr in low-glucose medium (see Figure S1 available online). The deacetylation of these transcriptional regulators could also be observed in mouse gastrocnemius muscle after overnight fasting (Figure 1D). Remarkably, these changes were rapidly reversed by reincubating myotubes in 25 mM glucose or refeeding mice (Figures 1A–1D and Figure S1). Hence, the activity of metabolic sensors and the acetylation levels and activity of SIRT1 targets coordinately respond to external nutrient availability.

We then explored the sequential events leading to the adaptations in glucose restriction. AMPK and ACC phosphorylation increased 18–24 hr after incubation in low glucose, in parallel to a decrease in mTOR signaling (Figure 1E). Changes in PGC-1α acetylation levels took place 30–36 hr after incubation in low-glucose medium (Figure 1B), in the absence of changes in SIRT1 protein levels (Figure 1E). Intracellular NAD^+ levels increased 30–36 hr after glucose restriction, in line with the timing for PGC-1α deacetylation (Figure 1F). These results indicate that changes in AMPK activity during glucose restriction precede changes in NAD^+ levels and SIRT1 activity.

Based on the above results, we tested whether AMPK could act as the primordial sensor triggering SIRT1-dependent metabolic adaptations. For this purpose, we overexpressed lacZ (control), wild-type (WT), or dominant-negative (DN) forms of $AMPK\alpha_1$ and examined the activation of PGC-1α in response to glucose deprivation. The DN-$AMPK\alpha_1$ robustly prevented AMPK activity (Figure 2A) and impaired PGC-1α deacetylation upon glucose restriction (Figure 2B). Consistent with the impaired deacetylation, studies on the mouse PGC-1α promoter reporter, which itself is activated in a PGC-1α-dependent manner, revealed that PGC-1α transcriptional activity was not significantly enhanced in glucose-restricted cells when AMPK activity was blunted (Figure 2C). Interestingly, DN-$AMPK\alpha_1$ did not prevent the transcriptional activity of a PGC-1α mutant where the 13 acetylable lysines are mutated to arginine (R13) (Figure 2C). This latter observation suggests that the phosphorylation of PGC-1α by AMPK (Jager et al., 2007) might act as a signal for deacetylation but does not contribute to the intrinsic activity of this transcriptional cofactor.

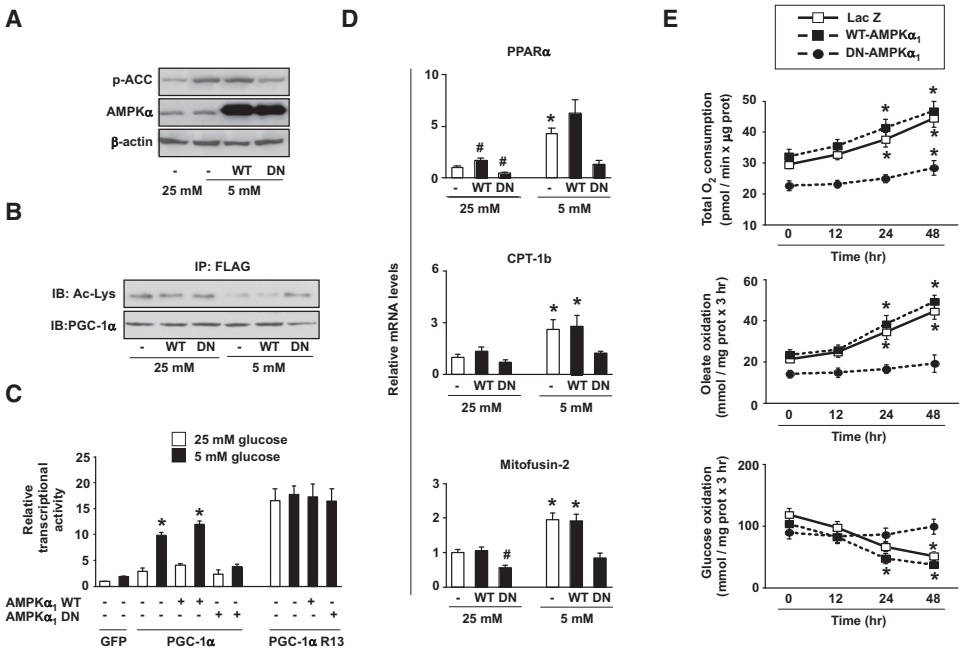

Figure 2. Disruption of AMPK Activity Renders PGC-1α Activity and Metabolic Adaptations Unresponsive to Glucose Restriction

(A) C2C12 myotubes were infected with adenovirus encoding FLAG-HA-PGC-1α and either lacZ, a WT, or a DN form of AMPKα$_1$. After infection, cells were left for 48 hr in 25 or 5 mM glucose medium. Of total extracts, 100 μg was used for western analysis.

(B) As in (A), but 500 μg from total extracts were used for immunoprecipitation against FLAG to test PGC-1α acetylation levels.

(C) C2C12 myoblasts were transfected with lacZ and either an empty vector (data not shown) or a luciferase reporter construct linked to the mouse PGC-1α promoter. Simultaneously, cells were infected with adenovirus encoding for GFP (–), FLAG-HA-PGC-1α, FLAG-HA-PGC-1α R13, WT-AMPKα$_1$, or DN-AMPKα$_1$ as indicated. Thirty-six hours later, cells were incubated for 48 hr in medium containing either 25 or 5 mM glucose, and luciferase activity was measured. A representative assay of four independent experiments is shown. Values are expressed as mean ± SE. * indicates statistical difference versus corresponding 25 mM glucose group.

(D) Similar to (A), but total mRNA was obtained for qRT-PCR analysis. Relative mRNA levels are shown as mean ± SE from four experiments. * indicates statistical difference versus corresponding 25mM glucose group. # indicates statistical difference versus lacZ-infected 25 mM group.

(E) C2C12 myotubes were infected as in (A). Then cells were left in 25 or 5 mM glucose medium. At the indicated time points, O_2 consumption, oleate, and glucose oxidation rates were measured. Results are shown as mean ± SE from 12, 6, and 4 independent experiments, respectively. * indicates statistical difference versus time = 0 hr.

In line with the defective PGC-1α activation during glucose restriction, the DN-AMPKα$_1$ prevented the increase in the mRNA levels of mitochondrial and fatty acid oxidation genes, such as PPARα, CPT-1b, mitofusin-2 (Figure 2D), ERRα, PDK4, and COXIV (Figure S2). We next evaluated the physiological impact of these changes by analyzing how oxidative metabolism responded to glucose restriction when AMPK activation is impaired. O_2 consumption gradually increased 24 and 48 hr after glucose restriction in lacZ- and WT-AMPKα$_1$-expressing myotubes (Figure 2E). This response was largely attenuated in cells expressing DN-AMPKα$_1$ (Figure 2E). While total O_2 consumption in lacZ- and WT-AMPKα$_1$-expressing myotubes increased 50.9% ± 9.7% and 45.5% ± 9.9%, respectively, after 48 hr in LG medium, only a 25.1% ± 5.7% increase was detected in the presence of DN-AMPKα$_1$ (Figure 2E). These differences were more evident when lipid oxidation was specifically examined. Even 48 hr after the initiation of glucose restriction, fatty acid oxidation rates were unaltered in myotubes expressing DN-AMPKα$_1$, in sharp contrast with myotubes expressing either lacZ or WT-AMPKα$_1$ (Figure 2E). Furthermore, impaired AMPK activation prevented the decrease in glucose oxidation rates observed in lacZ- and WT-AMPKα$_1$-expressing myotubes (Figure 2E). Hence, AMPK activation during energy stress allows a rapid shift from glucose to lipid as the main substrate for oxidation.

To provide in vivo evidence to support the above results, we used mice deficient in the AMPKγ$_3$ subunit (Barnes et al., 2004) The γ$_3$ is the predominant γ-isoform in glycolytic skeletal muscle (Mahlapuu et al., 2004) and is critical for effective adaptations of glycolytic muscle to metabolic challenges (Barnes et al., 2005b; Long et al., 2005). Fasting-induced PGC-1α and FOXO1 deacetylation was evident in EDL muscles from WT mice but severely blunted in AMPKγ$_3$ KO mice (Figure 3A). This defective SIRT1 activity could be consequent to the fact that intramuscular levels of NAD^+ were unresponsive to fasting in AMPKγ$_3$ KO mice (Figure 3B). Notably, muscle glycogen levels in WT mice dropped ~35% (~5 mmols × kg^{-1}) after fasting, while ~55% (~7 mmols × kg^{-1}) in the AMPKγ$_3$ KOs (Figure 3C), suggesting that the AMPKγ$_3$ KOs cannot shift to noncarbohydrate energy sources as efficiently as their WT littermates. Consequent to reduced PGC-1α activation, the expression of PGC-1α target genes was also impaired upon fasting in AMPKγ$_3$ KO mice (Figure 3D). Interestingly, fasting induced Nampt expression, but this increase was blunted in the AMPKγ$_3$

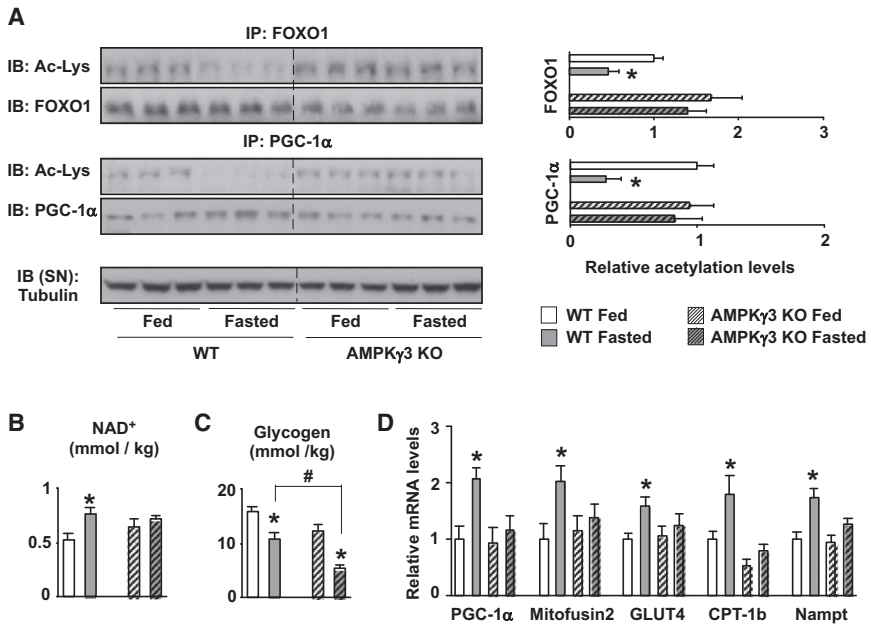

Figure 3. Impaired PGC-1α Deacetylation and Transcriptional Response to Fasting in AMPKγ_3 Kos

WT and AMPKγ_3 KO mice in fed or fasted (20 hr) state were sacrificed, and muscles were extracted and frozen.

(A) Of total protein extracts from EDL muscles, 2 mg was used to immunoprecipitate FOXO1 and PGC-1α and check their acetylation. Relative quantifications of FOXO1 and PGC-1α acetylation levels are shown on the right as mean ± SE of six muscles/group.

(B) Acidic extracts from 50 mg of gastrocnemius muscle were used to measure NAD^+ content. Results are shown as mean ± SE from six muscles measured in duplicate.

(C) Quadriceps muscle (20 mg) was used to measure glycogen content. Results are shown as mean ± SE from five muscles. # indicates statistical difference between the groups indicated.

(D) Total mRNA was extracted from quadriceps muscles and used for qPCR analysis. Results are shown as mean ± SE from ten muscles/group. Through the figure, * indicates statistical difference versus respective fed group.

KOs (Figure 3D), providing an additional explanation for the lack of changes in NAD^+ levels. Next, we tested whether administration of a claimed SIRT1 activator, such as resveratrol (Rsv), could rescue the defective response of PGC-1α acetylation to fasting in the EDL muscles of AMPKγ_3 KOs. Interestingly, Rsv efficiently decreased PGC-1α acetylation levels in EDL muscles from WT mice (Figure S3) but was unable to do so in muscles from the AMPKγ_3 KOs (Figure S3), indicating that the actions of Rsv on PGC-1α deacetylation require intact AMPK activity.

Finally, we evaluated whether AMPK was required for PGC-1α deacetylation in response to exercise, the most common energy challenge in skeletal muscle. PGC-1α and FOXO1 were robustly deacetylated 2.5 hr after swimming, an effect that was partially blunted in the AMPKγ_3 KO mice (Figure 4A). As observed in fasted muscle, the increase in NAD^+ observed during exercise recovery in WT mice was absent in AMPKγ_3 KOs (Figure 4B). A recent report indicated that increases in NAD^+ and SIRT1 activity after exercise could also derive from an increase in Nampt (Costford et al., 2009). Noteworthily, Nampt induction after exercise was impaired in AMPKγ_3 KO mice (Figure 4C), further indicating that AMPK controls Nampt expression (Figure 4C). Also similar to what was observed in fasting, glycogen levels were markedly lower in the AMPKγ_3 KO mice 2.5 hr after exercise (Figure 4D). This is in line with previous findings (Mu et al., 2003), indicating an impaired ability to rely on fat oxidation to recover glycogen levels.

DISCUSSION

We report that the acetylation levels of two SIRT1 targets, the transcriptional regulators PGC-1α and FOXO1, dynamically respond to energy stresses, such as exercise and fasting in skeletal muscle, or changes in glucose availability in C2C12 myotubes. Furthermore, our results indicate that AMPK activation precedes and determines the changes in SIRT1 activity in situations of energy stress. The regulation of the acetylation levels of transcriptional regulators through the AMPK/SIRT1 axis provides a mechanism by which mitochondrial and lipid oxidation genes can be rapidly and selectively controlled in response to energy levels.

Genetic ablation of the AMPKγ_3 subunit largely prevents physiological and/or pharmacological activation of AMPK (Barnes et al., 2005a) and was enough to impair fasting- and exercise-induced PGC-1α deacetylation. This unequivocally proves that SIRT1-mediated deacetylation of PGC-1α requires functional AMPK. The unchanged NAD^+ levels after fasting or during exercise recovery in the AMPKγ_3 KO mice suggest that these mice are metabolically inflexible and, together with the defective induction of Nampt, might explain the defective SIRT1 activation. Along the same line, the AMPKγ_3 KO mice showed higher glycogen depletion on fasting and after exercise. Our results reinforce previous observations showing that AMPK-defective mice have impaired glycogen metabolism (Barnes et al., 2005a; Fujii et al., 2007; Mu et al., 2003). Additionally, AMPKγ_3 KO mice highly rely on glucose metabolism during exercise recovery (Barnes et al., 2005a), explaining why glycogen levels hardly recover after exercise. Similarly, myotubes expressing a DN-AMPKα_1 highly depend on glucose as oxidative energy source even during glucose restriction. Altogether, these data illustrate that AMPK is necessary to efficiently shift between different energy substrates in the muscle and optimize the use of glycogen stores.

Noteworthily, while several models of AMPK deficiency display decreased basal mitochondrial gene expression (Jorgensen et al., 2005), lower voluntary exercise activity (Mu et al., 2001), and lower exercise tolerance (Fujii et al., 2007), defective AMPK does not robustly affect exercise-induced gene expression (Barnes et al., 2005b; Jorgensen et al., 2005). In line with this, AMPK acts on mitochondrial gene expression through PGC-1α (Jager et al., 2007), which itself is dispensable for mitochondrial gene induction after exercise (Leick et al., 2008). Contrastingly, AMPK is required for mitochondrial

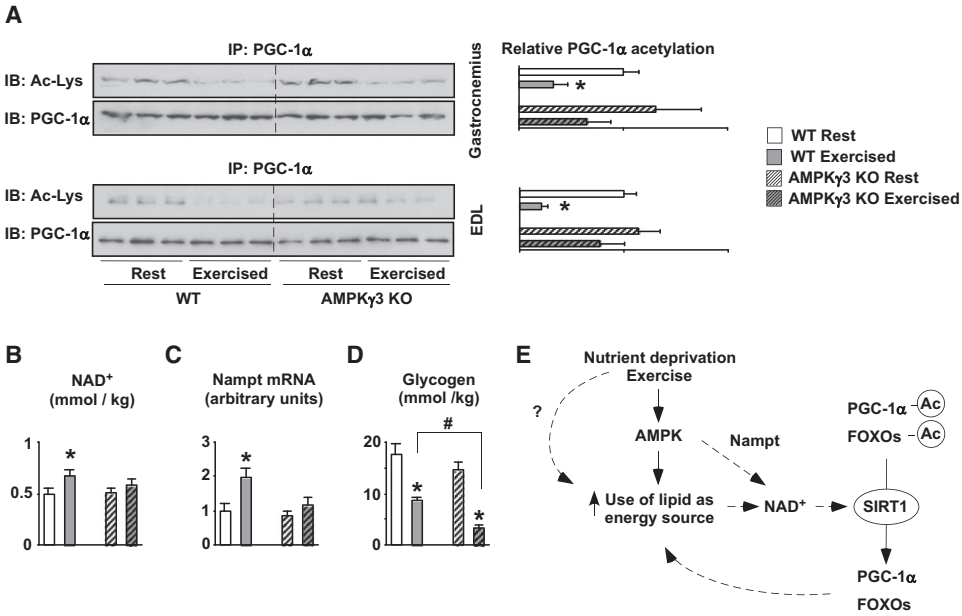

Figure 4. Impaired PGC-1α Deacetylation in Response to Exercise in AMPKγ3 Kos

(A) WT and AMPKγ3 KO mice were sacrificed at rest or 2.5 hr after swimming (see the Experimental Procedures), and muscles were frozen. Nuclear extracts (300 μg) from gastrocnemius or 2 mg of total proteins from EDL muscles were used to immunoprecipitate PGC-1α and check its acetylation levels. Quantifications of PGC-1a acetylation levels are shown on the right as mean ± SE.

(B) Acidic extracts from 50 mg of gastrocnemius were used to measure NAD^+. Results are shown as mean ± SE from three to four muscles per group measured in duplicate.

(C) Total mRNA from quadriceps was used to measure Nampt expression.

(D) Quadriceps muscle (20 mg) was used to measure glycogen content. Results are shown as mean ± SE from three to four muscles per group measured in duplicate. # indicates statistical difference between the groups indicated.

(E) Proposed scheme for the coordinated regulation of lipid utilization during energy stress. Upon low nutrient availability or increased energy demand, as during exercise, there is an increase in the AMP/ATP ratio, which activates AMPK, enhancing lipid oxidation in the mitochondria and inducing Nampt levels. These events will raise intracellular NAD^+ levels, triggering SIRT1 activation, which deacetylates PGC-1α and FOXOs, both of which regulate genes that further favor mitochondrial respiration and lipid mobilization. Through the figure, * indicates statistical difference versus respective fed group.

biogenesis upon pharmacological energy depletion (Zong et al., 2002). This reflects how exercise triggers more pathways than those derived from energy depletion, which redundantly regulate exercise-induced transcriptional changes (Jensen et al., 2009).

The clear effect of the ablation of AMPKγ3 on fasting-induced PGC-1α responses is striking, as AMPK activation during fasting is controversial (de Lange et al., 2006). AMPK activity, however, was estimated to be ~50% lower in the AMPKγ3 KOs than in WT mice after fasting (Barnes et al., 2005a). An additional explanation for the defects observed lies in the fact that NAD^+ levels remain stable during fasting/feeding transitions in the AMPKγ3 KO mice, probably due to the inability of their mitochondria to shift between substrates (Barnes et al., 2005a), which would prevent activation of SIRT1. This link between AMPK and mitochondrial fitness might also explain why AMPK participates in the adaptations to other situations such as calorie restriction (Apfeld et al., 2004; Schulz et al., 2007).

From a pharmacological perspective, our results on incubated muscle show that Rsv, initially believed to be a direct SIRT1 activator, requires AMPK to promote PGC-1α deacetylation in glycolytic muscle. Together with recent data (Um et al., 2009), our results suggest that SIRT1 activation in response to Rsv, rather than being a direct effect on SIRT1, might be an indirect consequence of AMPK activation (Canto et al., 2009). Another explanation is that phosphorylation by AMPK might be required for PGC-1α deacetylation by SIRT1 (Canto et al., 2009). Consequently, PGC-1α would remain acetylated when AMPK activity is defective even when SIRT1 is active. Altogether, these observations indicate that Rsv requires AMPK for its metabolic effects.

Based on our data, we propose that AMPK acts as an initial sensor of energy stress and allows the cell to efficiently shift between different energetic substrates. This, together with the ability of AMPK to regulate Nampt expression, impacts on intracellular NAD^+ levels, which modulate SIRT1 action on its downstream targets such as PGC-1α and FOXO1 (Figure 4D). Consequently, SIRT1 activation constitutes an indirect consequence of the metabolic and transcriptional rearrangements induced by AMPK activation.

EXPERIMENTAL PROCEDURES

Reagents and Materials

A full list of reagents and materials is provided in the Supplemental Experimental Procedures.

Animal Studies

For the fasting experiment in Figure 1, animals were purchased from Charles River (L'Arbresle, France). Ten-week-old C57Bl/6J male mice were maintained in a temperature-controlled (23°C) facility with a 12 hr light/dark cycle. To study

the effects of fasting, food was removed for 16 hr, including the overnight period. The AMPKγ_3 KO mice have been described previously (Barnes et al., 2004). WT or AMPKγ_3 KO mice (6–9 months old) had free access to water and standard rodent chow. When examining the effects of fasting, food was removed 20 hr prior to the extraction of hindlimb muscles, which were cleaned of fat and blood and quickly frozen in liquid nitrogen. For exercise experiments, mice swam in 45cm Ø plastic containers for four 30 min intervals separated by 5 min rest periods for a total swimming time of 2 hr. Then mice were dried and allowed to recover from exercise for 2.5 hr. In the recovery period mice had free access to food and water.

Cell Culture and Adenoviral Infection

C2C12 skeletal muscle cells were grown and differentiated as described (Canto et al., 2009). C2C12 were considered as myotubes after 96 hr of differentiation. Differentiation medium was supplemented with BSA-conjugated oleic acid (0.2 mM). Adenoviral infections of C2C12 myocytes were performed after 48 hr of differentiation. Cells were washed with PBS and left for 1 hr in serum-free DMEM 4.5g/l glucose containing the appropriate amount of viral particles (moi = 100 per each virus used, using GFP (for the different PGC-1α forms) or lacZ (for AMPKα_1 WT and DN forms) as control to make even the final viral amount). Media was replaced with fresh differentiation media for an additional 48 hr before any treatment took place. All the adenoviruses used in this study have been described (Rodgers et al., 2005; Woods et al., 2000).

Metabolic Measurements

Oxygen consumption was measured using the XF24 equipment (Seahorse Bioscience Inc., North Billerica, MA) as described (Watanabe et al., 2006). Oleate and glucose oxidation rates were estimated as described previously (Pich et al., 2005).

NAD^+ and NADH Measurements

NAD^+ intracellular levels in muscle (50 mg) and cultured myotube samples (1 mg) were estimated as described (Rodgers et al., 2005).

Statistics

Differences between two groups were assessed using two-tailed t tests. Analysis of variance, assessed by Bonferroni's multiple comparison test, was used when comparing more than two groups.

Additional experimental procedures can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cmet.2010.02.006.

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