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Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells

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Abstract

We examined in HepG2 cells whether glucose-induced changes in AMP-activated protein kinase (AMPK) activity could be mediated by SIRT1, an NAD-dependent histone/protein deacetylase that has been linked to the increase in longevity caused by caloric restriction. Incubation with 25 vs. 5 mM glucose for 6 h concurrently diminished the phosphorylation of AMPK (Thr 172) and ACC (Ser 79), increased lactate release, and decreased the abundance and activity of SIRT1. In contrast, incubation with pyruvate (0.1 and 1 mM) for 2 h increased AMPK phosphorylation and SIRT1 abundance and activity. The putative SIRT1 activators resveratrol and quercetin also increased AMPK phosphorylation. None of the tested compounds (low or high glucose, pyruvate, and resveratrol) significantly altered the AMP/ATP ratio. Collectively, these findings raise the possibility that glucose-induced changes in AMPK are linked to alterations in SIRT1 abundance and activity and possibly cellular redox state.

Keywords

AMPK; redox state; SIRT1; resveratrol

Introduction

AMP-activated protein kinase (AMPK) belongs to a family of highly conserved serine kinases that are regulated by nutritional and metabolic stresses that alter cellular energy state [1–3]. When activated, AMPK protects the cell against ATP depletion by stimulating processes such as fatty acid oxidation that promote ATP generation and inhibiting others, such as protein and lipid synthesis, that require ATP but are not acutely necessary for survival [1,4]. Although the activation of AMPK appears to be a direct consequence of an increase in the AMP to ATP ratio in many situations, studies in various tissues have shown that AMPK can be activated or inhibited by mechanisms that may not involve changes in adenine nucleotide levels [5,6]. In

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one such study, Itani *et al.* [7] demonstrated that the incubation of rat extensor digitorum longus (EDL) muscle with a high glucose medium (25 vs. 6 or 0 mM) for 4 hours diminished AMPK activity without changing the whole-tissue concentrations of creatine phosphate or adenine nucleotides. The reduced AMPK activity correlated with an increased release of lactate by the EDL, raising the possibility that alterations in its redox state contributed to these changes.

Sirtuins are a family of redox-sensitive, NAD^+-dependent deacetylases that regulate gene expression by controlling the acetylation status of lysine residues on histones, transcription factors, and transcriptional coactivators [8]. Sir2 and its mammalian homolog SIRT1 are induced in response to nutrient deprivation and are thought to mediate the effects of caloric restriction on longevity [9,10]. Rodgers *et al.* [11] found an increase in SIRT1 abundance and activity in the livers of 24-hour fasted mice, demonstrating increased deacetylation and activation of PGC-1α (PPAR-gamma coactivator 1-α). Conversely, refeeding following a 24-hour fast decreased hepatic SIRT1 abundance, as did incubation of cultured Fao rat hepatocytes in a high glucose medium (10 vs. 0 mM). We have previously shown that refeeding following a 48-hour fast similarly reduces AMPK activity in rat liver [12], as does incubation of HepG2 cells with 25 vs. 5 mM glucose [3]. These findings suggest a link between SIRT1 and AMPK.

In the present study, the linkage between SIRT1 and AMPK was examined more directly. We determined in HepG2 cells whether 1) glucose- and pyruvate-induced changes in AMPK activity (phosphorylation) are associated with alterations in SIRT1 abundance and activity, 2) SIRT1 activation and inhibition by pharmacological agents produce parallel changes in AMPK, and 3) observed alterations in AMPK activity occur in the presence or absence of changes in cellular energy state. The results suggest concurrent regulation of SIRT1 and AMPK in the absence of a change in whole cell energy state.

Materials and Methods

Resveratrol was from Calbiochem (San Diego, CA). Pyruvate and nicotinamide were from Sigma (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and PBS were from Gibco (Grand Island, NY). Rabbit monoclonal anti-phospho-Thr 172 AMPK and rabbit polyclonal anti-AMPKa subunit antibodies were from Cell Signaling. Anti-phospho-Ser 79 ACC1 was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-SIRT1 (H-300) and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments

HepG2 cells were cultured in DMEM containing 5 mM glucose supplemented with 10% FBS, 1% penicillin/streptomycin and subjected to assays after overnight serum and pyruvate depletion. C2C12 myocytes were cultured to 80% confluence in 5mM glucose DMEM containing 1% GlutaMAX, 1% penicillin/streptomycin and 10% FBS, then differentiated to 80% myotubes in 5 mM glucose DMEM containing 2% Horse Serum, 1% GlutaMAX, and 1% penicillin/streptomycin.

Immunoblotting analysis

Samples (50 ug protein, as determined by Bio-Rad assay) were separated by SDS-PAGE, transferred to PVDF membrane, blocked with 5% milk in TBST, and incubated with primary antibody overnight at 4°C. Bound antibodies were detected with HRP-linked secondary antibodies and visualized using enhanced chemiluminescence (Thermo Fisher) and autoradiography.

PGC-1α acetylation assay

PGC-1α lysine acetylation was determined in HepG2 cells transfected with an HA-tagged PGC-1α adenovirus, as described previously [13].

Other analyses

ATP, AMP, and ADP were measured spectrophotometrically as described previously [14]. Lactate release was determined spectrophotometrically using lactate dehydrogenase and NAD [15].

Experimental Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained on a 12-h light cycle and fed ad libitum. Experiments were performed following an 18–20 h fast. All research on these animals was reviewed and performed in accordance with the requirements of the IACUC at Boston University Medical Center.

Muscle incubation

Rats were anesthetized with sodium pentobarbital (6mg/100g body weight i.p.). EDL muscle were isolated as described previously [14]. Muscles were initially equilibrated in Krebs-Henseleit solution containing 5 mM glucose for 20 minutes, followed by incubation ± resveratrol in DMSO for 2 h.

Statistics

Results are expressed as means ± SE. Student's t-test or ANOVA followed by Student-Newman-Keuls post hoc analysis were used to determine significance ($p<.05$).

Results

Effect of incubation with different glucose concentrations on the phosphorylation of AMPK and ACC, and SIRT1 protein abundance and activity

Using a high glucose-induced model of insulin resistance in HepG2 cells [3], we first confirmed the finding of Zang *et al.* [3] that incubation of HepG2 cells in a high glucose medium (25 vs. 5 mM) for 6 hours decreases the phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) without altering AMPK abundance (Fig 1A, B). We found that these alterations were not paralleled by changes in cellular energy state, as reflected by the AMP/ATP ratio (Table 1). On the other hand, they were associated with a decrease in SIRT1 protein abundance and a more than 2-fold increase in PGC-1α acetylation (Fig. 1A–D), suggesting that hyperglycemia decreased SIRT1 activity. In addition, the release of lactate was increased by 23% in the cells incubated with 25 vs. 5 mM glucose for 6 hours ($n=3$; $p<0.01$).

The effects of pyruvate on AMPK and ACC phosphorylation, and SIRT1 protein and activity

The effects of incubating HepG2 cells in a 5 mM glucose medium with increasing concentrations of pyruvate are shown in Figure 2. The addition of 0.1 or 1 mM pyruvate markedly enhanced the phosphorylation of AMPK, and even more so that of ACC at 2 hours (Fig. 2A, B). In cells incubated with 1 vs. 0 mM pyruvate, these changes were associated with a 30% increase in SIRT1 protein abundance (Fig. 2B) and a 60% decrease in the acetylation of PGC-1α (Fig. 2C, D). Incubation with 1 mM pyruvate did not increase the AMP/ATP ratio (Table 1), suggesting that the increase in AMPK activity occurred without an alteration of cellular energy state.

Nicotinamide suppresses SIRT1 activity and phosphorylation of AMPK and ACC

Nicotinamide and the reduced dinucleotide, NADH, are inhibitors of sirtuins [16], although their biological significance is incompletely understood. Rodgers *et al*. [11] have shown that nicotinamide induces PGC-1α acetylation in 293T cells, an effect that is overcome by SIRT1 overexpression. Here we demonstrate that HepG2 cells exposed to 10 mM nicotinamide for 6 hours show enhanced acetylation of PGC-1α (Fig. 4A, B) as well as diminished phosphorylation of AMPK and ACC (Fig. 4C, D). Thus, nicotinamide concurrently modulates the activities of SIRT1 and AMPK.

SIRT1 activators enhance the phosphorylation of AMPK in HepG2 and C2C12 cells, and in incubated rat EDL muscle

Resveratrol, a polyphenol found in foods such as grapes, red wine, and peanuts, has received increasing attention as a putative SIRT1 activator that can mimic the effects of caloric restriction on the aging process [17], the development of obesity and insulin resistance in fat-fed rodents [13,18], and possibly the prevalence of atherosclerotic cardiovascular disease in humans [19]. Fig. 4A and B demonstrate that resveratrol increases the phosphorylation of AMPK in a dose dependent manner in HepG2 cells. The dose response of ACC phosphorylation was less apparent, although at all concentrations resveratrol significantly increased p-ACC ($p<0.05$). A time course experiment demonstrated that at a concentration of 100 μM, a marked increase in p-AMPK and p-ACC was evident within 5 minutes (Fig. 4C). No alteration in SIRT1 abundance was observed (Fig. 4A), in keeping with evidence suggesting that resveratrol binds to and activates SIRT1 by increasing its protein substrate affinity [20]. Similar effects of resveratrol were observed in C2C12 cells and incubated rat extensor digitorum longus (EDL) muscle, in which incubation with 50 and 100 μM resveratrol enhanced p-AMPK and p-ACC abundance (data not shown). Resveratrol had no effect on the cellular energy state of HepG2 cells after incubations of 1 hour (Table 1) or 15 min (data not shown). Quercetin, a putative SIRT1-activating flavonoid, also enhanced the phosphorylation of AMPK and ACC in a dose-dependent manner in HepG2 cells (Fig. 4D). As with resveratrol, no change in the abundance of SIRT1 was observed.

Discussion

The results indicate that incubation in a high glucose medium (25 vs. 5 mM) diminishes the activity of AMPK in HepG2 cells, whereas incubation with pyruvate has the opposite effect. The observed changes in AMPK activity were not associated with alterations in cellular AMP/ATP ratios, but were linked to concomitant changes in SIRT1 abundance and activity. In line with this, we found that AMPK phosphorylation is diminished during incubation with nicotinamide, a SIRT1 inhibitor, whereas resveratrol, a putative SIRT1 activator [17], enhanced the phosphorylation of AMPK without altering cellular energy state. Similar findings were obtained with another putative SIRT1 activator, quercetin [17], although energy state was not determined. The effects of resveratrol on p-AMPK were also observed in C2C12 myotubes and incubated rat EDL muscle, indicating that they are not tissue- or cell-specific. Overall these results provide strong correlative evidence for a linkage between SIRT1 and AMPK.

The observation that increasing the ambient glucose concentration decreases SIRT1 abundance is in agreement with the findings of Rodgers et al. [11] in Fao rat hepatocytes. Collectively, these findings raise the question of how changes in the ambient glucose concentration influence SIRT1. The sirtuins depend on the availability of NAD^+ as both an activator and a substrate for deacetylase reactions. Hence, changes in the NAD^+/NADH ratio within a cell, i.e. the cellular redox state, may influence SIRT1 activity. Results from the present study show that incubation in a high glucose medium increases the amount of lactate released by HepG2 cells. If as assumed this reflects changes in the cytoplasmic and nuclear free NAD^+/NADH ratio

[21], it raises the possibility that glucose-induced alterations in cellular redox state could influence the activity of SIRT1 and secondarily AMPK. This possibility is in line with the observation that in the fasted state, where both SIRT1 and AMPK are activated, hepatic pyruvate and NAD^+ content are increased whereas lactate content is decreased [11]. Furthermore, incubation with pyruvate, which acutely would increase the NAD^+/NADH ratio, has been shown to increase SIRT1 abundance in Fao cells [11], and in the present study it increased SIRT1 and AMPK activity in HepG2 cells. Furthermore, we found that incubation with quercetin, a SIRT1-activating compound that has been shown to increase the acetoacetate to β-hydroxybutyrate ratio [22] (an indicator of the mitochondrial NAD^+/NADH ratio), also increased AMPK activity. Collectively these data suggest a correlation between redox state and the activities of SIRT1 and AMPK; however, direct measures of cellular redox state, such as the lactate/pyruvate ratio and cellular NAD and NADH, are needed to establish this more definitively. Still unanswered is the basis for the changes in SIRT1 abundance observed in this and other studies[11]. In this context, Zhang *et al.*[23] have proposed that the redox-sensitive transcriptional corepressor, CtBP, may regulate SIRT1 transcription.

The notion that changes in SIRT1 may regulate AMPK activity is also supported by other recent studies. Overexpression of SIRT1 has been found to increase the phosphorylation of AMPK and ACC both in vivo and in vitro [24], by a reaction dependent on the AMPK kinase LKB1 [24–26]. More specifically, we found that SIRT1 deacetylates lysine residue 48 of LKB1, and that this is associated with LKB1 movement from the nucleus to the cytoplasm, where it binds to STRAD (STE-related adapter) and MO25 (mouse protein 25) and is activated [26]. Likewise, Hou et al. [24] have found that pharmacologic and genetic inhibition of SIRT1 in HepG2 cells attenuates the increase in AMPK phosphorylation caused by polyphenol exposure, and it has been demonstrated that refeeding after a fast decreases SIRT1 [11], LKB1 and AMPK activity, and increases LKB1 acetylation in rodent liver [26].

The data presented in this report, together with several other recent studies [11,24,26], are consistent with a model in which alterations in the ambient glucose concentration influence SIRT1 and secondarily AMPK activity. In contrast, an alternative model to explain the effects of glucose concentration on SIRT1 has recently been proposed by Fulco et al. [27]. In brief they reported that glucose deprivation (5 vs. 25 mM) inhibited the differentiation of C2C12 myoblasts by leading to a decrease in cellular ATP and activation of AMPK, which in turn caused the activation of SIRT1. They also demonstrated that SIRT1 activation was enhanced by the AMPK activator AICAR, and that both glucose deprivation and AICAR induced transcription of the NAD^+ biosynthetic enzyme Nampt (nicotinamide phosphoribosyltransferase), resulting in a decrease in the concentration of the SIRT1 inhibitor nicotinamide, an increase in the NAD^+/NADH ratio, and activation of SIRT1. These findings suggest that the upstream event in glucose regulation of AMPK is a change in energy state, a finding also observed in pancreatic β-cells [28]. In contrast, in the present study as well as in earlier studies in incubated rat EDL muscle (30 min - 2 h) [7] and human umbilical vein endothelial cells (2 h) [29], glucose deprivation led to AMPK activation in the apparent absence of a decrease in cellular energy state, although the possibility that a transient alteration in energy state occurred at earlier time points was not excluded. Thus, whether glucose regulates SIRT1 by effects on cellular energy or redox state or by both mechanisms remains to be determined. If both mechanisms operate, it would suggest the existence of a SIRT1/AMPK cycle that links cellular redox and energy states.

In summary, the results presented here support a strong association between SIRT1 and AMPK in mammalian cells, as demonstrated by concurrent regulation of both molecules in response to varying concentrations of glucose and pyruvate. Additionally, exposure to SIRT1 activators and inhibitors resulted in AMPK activation and inhibition, respectively. The absence of significant changes in energy state (where measured) suggest that modulation of SIRT1 by

redox changes could mediate the effects of glucose and pyruvate on its activity and abundance; however, additional studies are needed to establish this with greater certainty.

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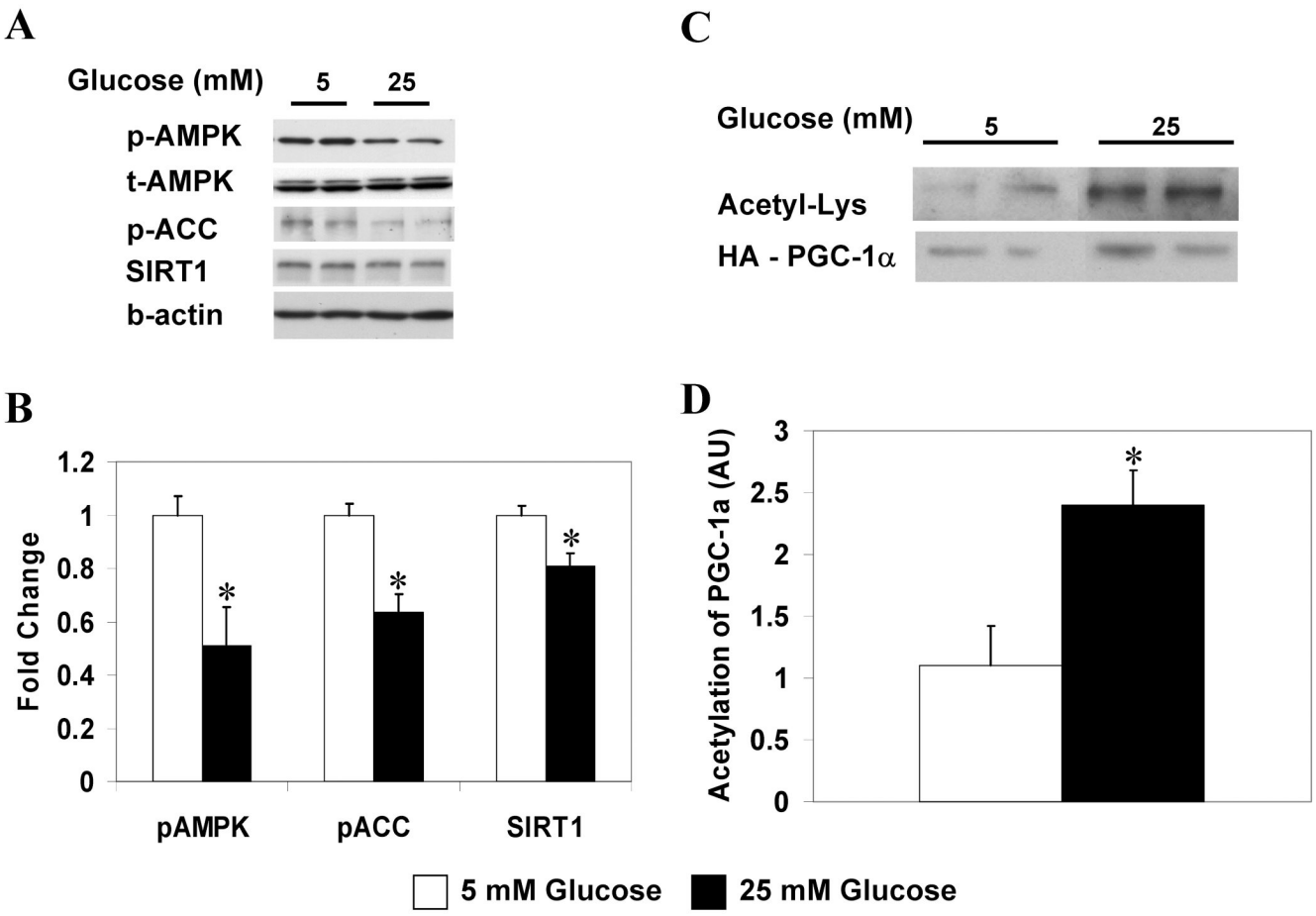

Figure 1. Increasing the ambient glucose concentration decreases the phosphorylation of AMPK and ACC, and the abundance and activity of SIRT1 in HepG2 cells

HepG2 cells incubated in 5 or 25 mM glucose for 6 h. (A) Western blot analysis for p-AMPK (Thr 172), p-ACC (Ser 79), and SIRT1. (B) Quantification of representative blot shown in A, results are means ± SE (n=5); *,p<0.05. (C) PGC-1α acetylation after 6 h incubation in 5 or 25 mM glucose. (D) Quantification of Acetyl-Lys/HA-PGC-1α. Results are means ± SE, (n = 4); *, $p<0.05$.

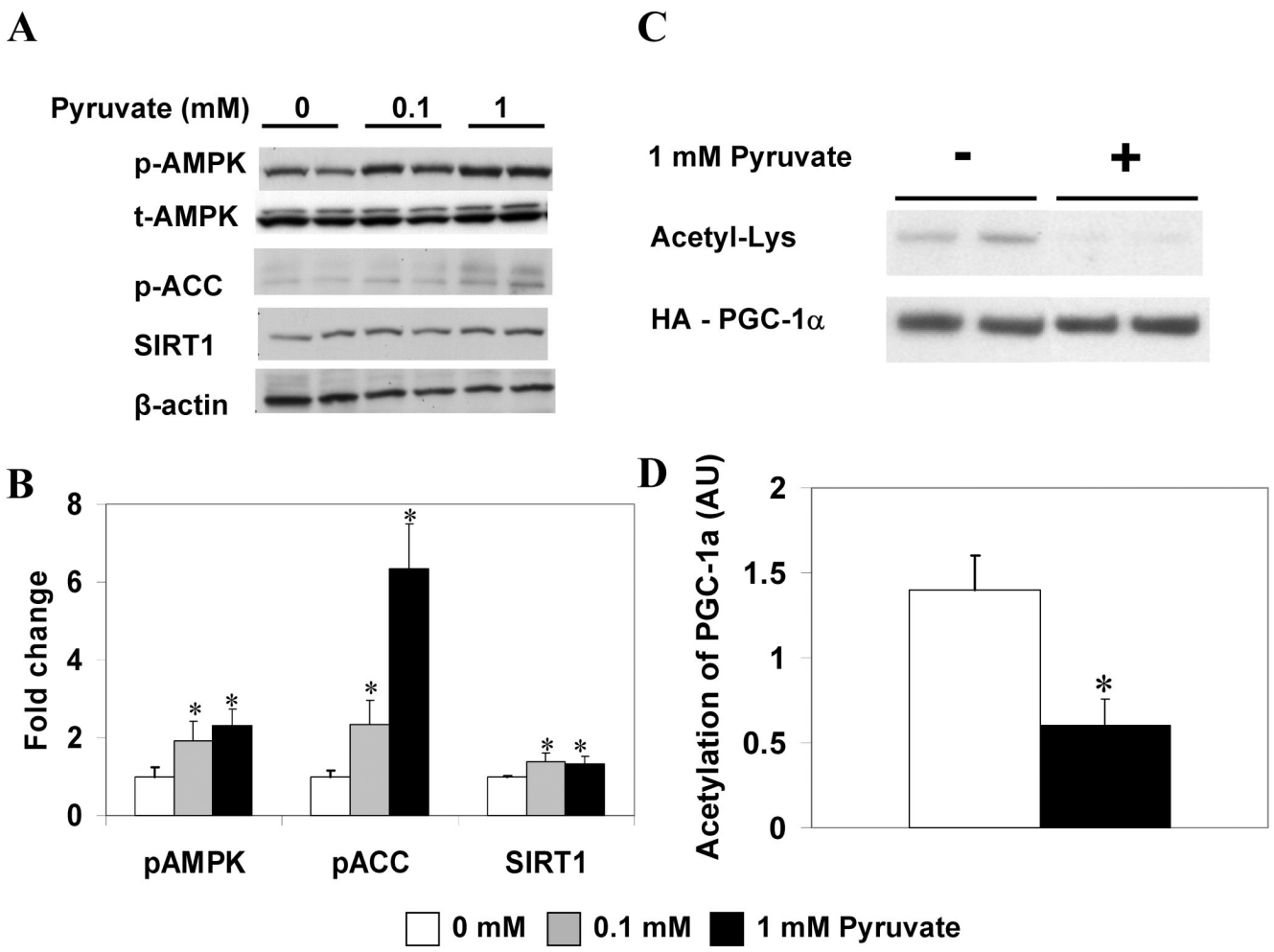

Figure 2. Increasing pyruvate concentration increases p-AMPK, p-ACC, and SIRT1 abundance and activity

(A) Western blot analysis of HepG2 cells following 2 h incubation with the indicated concentrations of pyruvate. (B) Quantification of p-AMPK, p-ACC, and SIRT1 relative to β-actin. Results are means ± SE (n = 6); *, $p<0.05$ vs. 0 mM. (C) PGC-1α acetylation after 2 h incubation ± 1 mM pyruvate. (D) Quantification of Acetyl-Lys/HA-PGC-1α. Results are means ± SE, (n = 4); *, $p<0.05$.

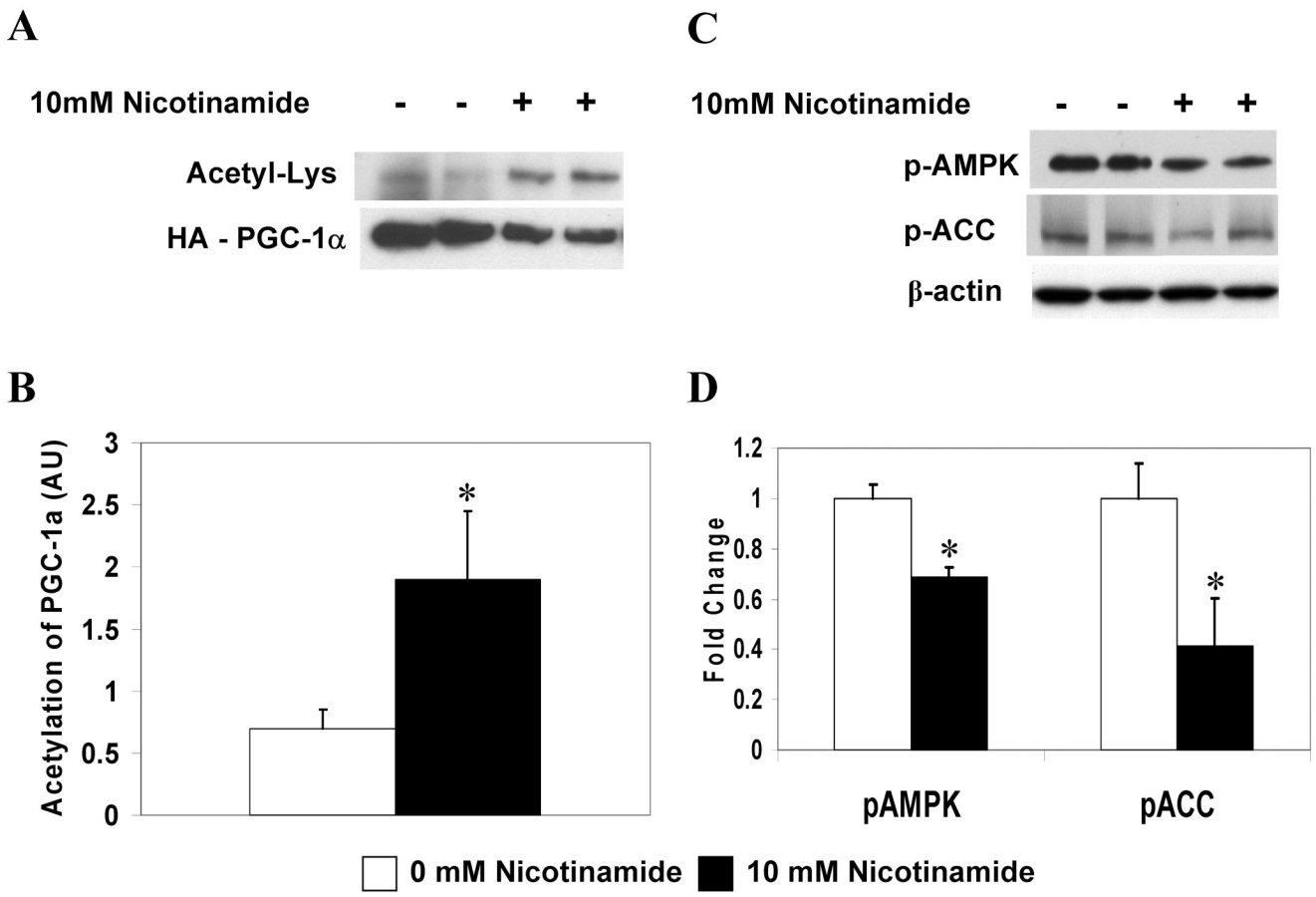

Figure 3. Nicotinamide increases PGC-1α acetylation and decreases the phosphorylation of AMPK and ACC

(A) PGC-1α acetylation in HepG2 cells incubated ± 10 mM nicotinamide (NAM) for 6 h. (B) Quantification of Acetyl-Lys/HA-PGC-1α. Results are means ± SE, (n = 4); *, $p<0.05$. (C) Western blot analysis of p-AMPK and p-ACC. (D) Quantification of representative blot shown in C. Results are means ± SE, (n = 4); *, $p<0.05$.

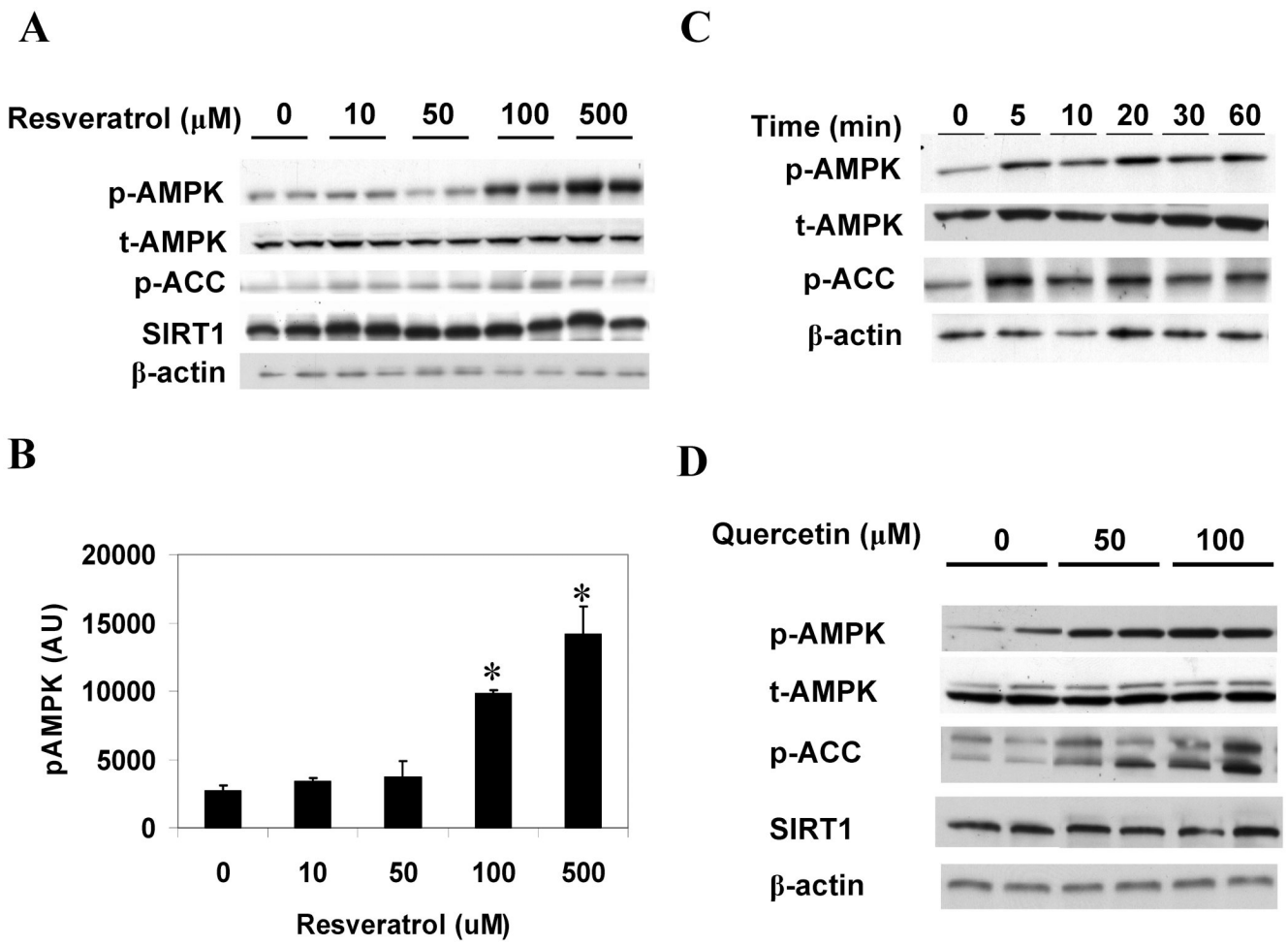

Figure 4. Resveratrol and quercetin increase the phosphorylation of AMPK and ACC in HepG2 cells

(A, B) Dose response of p-AMPK and p-ACC to 1 h resveratrol incubation. Results are means ± SE (n = 2); *,p<0.05 vs. 0 μM resveratrol. (C) Time course of changes in p-AMPK and p-ACC caused by 100 μM resveratrol. (D) Western blot analysis showing enhanced p-AMPK and p-ACC following 1 h incubation with increasing concentrations of quercetin.

Table 1

The effect of variations in glucose, pyruvate, and resveratrol concentration on cellular energy state in HepG2 cells

	ATP	ADP	AMP	AMP/ATP
		6 h incubation, nmol/mg protein		
5 mM glucose	9.4 ± 1.4	2.2 ± 0.2	0.39 ± 0.06	0.042 ± 0.009
25 mM glucose	9.7 ± 0.8	3.10 ± 0.1	0.31 ± 0.03	0.035 ± 0.003
		2 h incubation, nmol/mg protein		
0 mM pyruvate	11.9 ± 0.5	3.4 ± 0.2	0.37 ± 0.02	0.031 ± 0.001
1 mM pyruvate	12.6 ± 0.2	3.6 ± 0.1	0.34 ± 0.02	0.027 ± 0.002
		1 h incubation, nmol/mg protein		
0 uM resveratrol	11.3 ± 0.8	2.4 ± 0.2	0.28 ± 0.03	0.025 ± 0.002
50 uM resveratrol	11.7 ± 1.1	2.6 ± 0.2	0.36 ± 0.06	0.033 ± 0.009
100 uM resveratrol	11.9 ± 1.1	2.2 ± 0.2	0.39 ± 0.07	0.032 ± 0.005

Unless otherwise noted, incubations were carried out in 5 mM glucose DMEM. Adenine nucleotide levels were measured spectrophotometrically. Values are means ± SEM, n = 9 (n = 3/group on 3 independent experiments)