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The AMPK-SIRT signaling network regulates glucose tolerance under calorie restriction conditions

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Abstract

SIRT1 and AMP protein kinase (AMPK) share common activators, actions and target molecules. Previous studies have suggested that a putative SIRT1-AMPK regulatory network could act as the prime initial sensor for calorie restriction-induced adaptations in skeletal muscle - the major site of insulin-stimulated glucose disposal. Our study aimed to investigate whether a feedback loop exists between AMPK and SIRT1 in skeletal muscle and how this may be involved in insulin sensitivity and glucose tolerance. To investigate this we used skeletal muscle specific AMPK α 1/2 knockout mice (AMPK α 1/2^{-/-}) fed *ad libitum* (AL) or a 30% calorie restricted (CR) diet and L6 rat myoblasts incubated with SIRT1 inhibitor (EX527). CR-AMPK α 1/2^{-/-} displayed impaired glucose tolerance and reduced insulin sensitivity, in association with down-regulated SIRT1 and PGC-1 α expression. Moreover, AMPK activity was decreased following SIRT1 inhibition in L6 cells. This study demonstrates that skeletal muscle-specific AMPK deficiency impairs the beneficial effects of CR on glucose tolerance and insulin sensitivity, and that these effects may be dependent on reduced SIRT1 levels.

Key words

Akt; AMP protein kinase (AMPK); calorie restriction; glucose; insulin; SIRT1; PGC-1 α

Introduction

Downregulation of AMPK activity in response to high glucose, with a parallel decrease in SIRT1 activity was observed in cultured HepG2 cells [1, 2]. This was associated with increased lactate release, suggesting a decrease in the NAD⁺/NADH ratio, which likely contributed to the decrease in SIRT1 activity. These findings, together with the demonstration that SIRT1 and AMPK share common activators, actions and target molecules [3] led us to an examination of a possible link between SIRT1 and the AMPK in skeletal muscle. The precise interactions between SIRT1 and AMPK in skeletal muscle remain unclear, but given the function of skeletal muscle as the major site of glucose uptake and the known metabolic regulatory roles of SIRT1-AMPK, this signaling network is potentially of great importance. For example, AMPK has important acute metabolic actions in skeletal muscle, such as promotion of long chain fatty acids (LCFA) oxidation (lipid-lowering effect), glucose

uptake and glucose and glycogen breakdown (glucose-lowering effect) [4, 5, 6]. SIRT1 has been shown to play an important role in controlling glucose metabolism, insulin action, fat storage, and nutrient sensing [7, 8].

However, in marked contrast to the effects of AMPK, insulin suppresses fat oxidation and stimulates glycogen, lipid and protein synthesis and may often oppose the actions of AMPK [9, 10]. Interestingly, common actions of AMPK and insulin are also observed, in particular, stimulation of glucose uptake into skeletal muscle [11]. It has been recently established that AMPK acts as the prime initial sensor for fasting-induced adaptations in skeletal muscle and that SIRT1 downstream signaling is blunted in the absence of AMPK [12].

Caloric restriction (CR) has been shown to protect against onset age-related diseases [13, 14] and to reduce mortality linked to T2DM and cardiovascular diseases [15, 16]. The effects of CR have been linked to changes in activity of AMPK and SIRT1.

The research here described aims to understand the mechanisms by which AMPK and SIRT1 may interact to regulate insulin sensitivity in skeletal muscle during CR. To understand these signalling pathways, we utilized skeletal muscle-specific AMPK α 1/2 double knockout mice subjected to CR or starvation as manipulations to modify insulin sensitivity. In addition, L6 skeletal muscle myoblasts were used to determine regulatory interactions between AMPK and SIRT1.

Materials and Methods

Experimental Animals

Female skeletal muscle-specific AMPK α 1/2-knockout (AMPK^{-/-}) or wild-type control (WT) mice (kindly provided by Institute Cochin INSERM; Paris, France) were maintained on CR (CR-AMPK^{-/-}; WT-AMPK^{-/-}) or *ad libitum* (AL-AMPK^{-/-}; AL-AMPK^{-/-}) feeding regimes. CR was conducted between 2.5 – 4 months of age and lasted for 10 weeks. Food was adjusted on a daily basis to ensure that CR mice consumed 70% of the calories consumed by AL-fed mice (calculated on the average of food intake during the last week before starting the caloric restriction protocol). Mice were sacrificed in the immediate post-absorptive state or after 5 hours of fasting (all groups).

All mice were maintained on a 12:12-h light-dark cycle (light from 7am to 7pm), with controlled temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and air conditioned environment, and received standard high-carbohydrate rodent chow.

Mouse studies were reviewed and approved (agreement no. 75-886) by the Directeur Départemental des Services Vétérinaires of the Préfecture of Police of Paris and were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Intra-peritoneal glucose tolerance test (IPGTT)

To examine whole-body glucose homeostasis, mice (10/group) were fasted overnight, followed by intra-peritoneal injection of glucose (2 g/kg of body weight). Blood samples for glucose analysis were collected from the tail vein at 0, 20, 40, 60, 80 and 120 min after glucose injection. Blood glucose concentration was measured in whole blood with an automatic glucose monitor (One Touch Ultra, Lifescan; Johnson & Johnson, Milpitas, CA). Plasma insulin levels were measured in the blood samples collected from the tail vein at 0, 20 min during IPGTT, using a specific ELISA (Linco, St Charles, MO).

Insulin administration

To investigate whole body insulin sensitivity, mice (10/group) were administered insulin (1 Unit of insulin/kg of body weight; I.P) 5 minutes before sacrifice.

Cell culture

Cells were cultured at 37°C and 5% CO_2 , in DMEM containing 25mM of glucose, non-essential amino acids and supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM glutamate, 500 IU/ml penicillin and 100 μM streptomycin. For treatments, myoblasts were either maintained in DMEM (with FCS), as described above (termed 'fed cells') or in serum-free DMEM (without FCS; termed 'starved cells'). Fed and starved cells were incubated with SIRT1 inhibitor Ex527 (10 μM ; 3.5 h; Tocris Bioscience). Negative controls consisted of 1ml of media containing an equal concentration of DMSO.

Immunoblotting

Immunoblotting was conducted as previously described [17], using primary

antibodies listed in Supplemental Table 1. Reference protein measurements were made with mouse monoclonal anti- β -actin (clone AC-15; Sigma, Poole, UK) primary antibody.

Quantitative RT-PCR

All gene expression was measured by qRT-PCR, as previously described [18] using Taqman or Sybr green methodology. Gene expression was determined by $\Delta\Delta C_T$ normalised against 18S control ribosomal RNA. For primer and probe details see Supplemental Table 2.

Statistical analysis

Results are expressed as mean \pm SEM. Statistical comparisons were obtained using GraphPad (GraphPad Software, CA, USA). Statistical differences were calculated using a paired t-test, or two-way ANOVA followed by Bonferroni's Post-test where appropriate.

Results

Loss of AMPK α 1 and -2 subunits decreases AMPK protein expression in skeletal muscle of AL- and CR-fed mice

Skeletal muscle AMPK protein levels were decreased in AL- and CR-AMPK^{-/-} mice (Fig. 1A). Moreover, levels of AMPK activity, denoted by phosphorylation on Thr¹⁷², were reduced by 50% in skeletal muscle of AL- and CR-AMPK^{-/-} mice compared to AL-WT and CR-WT respectively (both $P < 0.005$).

Insulin treatment did not have any effect on AMPK activation (Fig. 1B).

Acetyl CoA Carboxylase (ACC) is a down stream target of AMPK, where by AMPK inhibits ACC activity through phosphorylation on Ser²¹⁸ [19]. AMPK^{-/-} had no effect on protein levels of total ACC in AL or CR groups (Fig. 1C), whilst ACC was phosphorylated at Ser²¹⁸ in skeletal muscle of AL-WT and AL-AMPK^{-/-} mice. Protein expression of phospho(Ser²¹⁸)ACC did not differ significantly between WT and AMPK^{-/-} mice and insulin treatment did not seem to have an effect on ACC protein expression (Fig. 1C and 1D).

Taken together, these data demonstrate that these mice are suitable for study of the effects of reduced skeletal muscle AMPK signalling.

AMPK α 1/2 knockout impairs CR-mediated improvements in whole-body glucose tolerance

AL-AMPK^{-/-} mice gained moderately more weight over 10 weeks compared to AL-WT mice. However, both CR-WT and CR-AMPK^{-/-} lost weight, although the absence of AMPK did not alter CR-mediated weight changes in the mice (Fig. 2A).

To assess the effects of the experimental diets and AMPK^{-/-} on whole body glucose tolerance and insulin secretion we utilized an IP glucose tolerance test (IPGTT). There were no significant differences in basal fasting plasma glucose and insulin levels between the four groups (Fig. 2B and 2D). However, CR-WT mice displayed improved glucose tolerance, where blood glucose levels were significantly lower 20 – 60 minutes post-glucose injection, compared to the other groups (**P*<0.05) (Fig. 2B and 2C). Notably, this effect was lost in CR-AMPK^{-/-} mice, demonstrating that skeletal muscle AMPK is required for mediating the beneficial effects of CR on glucose tolerance (**P*<0.05) (Fig. 2B and 2C). Interestingly, glucose levels peaked at 40 minutes-post glucose injections in CR-AMPK^{-/-}, but at 20 minutes post-glucose in AL-AMPK^{-/-} mice, perhaps indicating subtly different mechanisms of action. The early raised blood glucose levels were lost between 60 – 120 minutes, and blood glucose returned to basal levels after 120 minutes in all four groups (Fig. 2B). CR-WT mice did not display increased plasma insulin levels after 20 minutes compared to AL-WT and AL-AMPK^{-/-}, indicating that the improvements in glucose tolerance relative to these groups were likely mediated by increased peripheral insulin sensitivity, rather than elevated pancreatic insulin secretion (Fig. 2D). However, plasma insulin was slightly reduced in CR-AMPK^{-/-} mice compared with the other three groups, suggesting that AMPK deficiency in CR may lead to lower insulin secretion (Fig. 2D).

SIRT1, SIRT6 and SIRT3 gene expression are suppressed by AMPK deletion in skeletal muscle of AL fed mice

NAD⁺ biosynthetic activity of NAMPT results in activation of NAD⁺-dependent sirtuin enzymes, particularly SIRT1 [20]. AMPK has been reported to induce NAMPT with consequent raised NAD⁺ levels and increased SIRT1 activity [21]. We studied NAMPT gene expression in order to better understand the possible link AMPK-NAMPT-SIRT1 in CR. Loss of AMPK α 1/2 subunits led to a marked decrease in skeletal-muscle NAMPT mRNA expression in AL mice (<75% vs. AL-AMPK^{+/+}, ** P <0.01) (Fig. 3A). CR also suppressed skeletal-muscle NAMPT gene expression in WT mice (<75% vs. AL-AMPK^{+/+}, ** P <0.01). Skeletal-muscle NAMPT gene expression levels were comparable in AL-AMPK^{-/-} mice, CR WT mice and CR-AMPK^{-/-} mice (<75% vs. AL-AMPK^{+/+}, ** P <0.01). This data shows that deficiency in AMPK and CR separately lower skeletal muscle NAMPT expression, but AMPK deficiency does not lead to further NAMPT mRNA decrease in CR mice (Fig. 3.1A).

We next undertook to determine the relevance of the observed NAMPT/AMPK changes to sirtuin levels and function. SIRT1 has been reported to improve glucose uptake in skeletal muscle [7]. Loss of AMPK decreased SIRT1 mRNA expression in skeletal muscle of AL fed animals (Fig. 3.1B). However, in contrast, SIRT1 gene expression was unchanged in both CR-WT and CR-AMPK^{-/-} mice (Fig. 3.1B).

SIRT6, another nuclear sirtuin, reportedly enhances skeletal muscle insulin signaling and activation of Akt in mice, leading to hypoglycemia and associated increases in membrane association of GLUT1 and GLUT4, which enhances glucose uptake [22]. Skeletal-muscle SIRT6 gene expression was moderately suppressed by AMPK deficiency, both in AL-fed mice and CR mice (c.f. SIRT1 gene expression, where the effect of CR is blocked by AMPK deficiency) (Fig. 3.1C).

The mitochondrial sirtuin, SIRT3 has been reported to mediate CR-associated reduction of oxidative damage [23], as well as promoting fatty acid oxidation and insulin sensitivity in skeletal muscle. SIRT3 mRNA showed decreased gene expression in CR-AMPK^{-/-} mice when compared to CR-WT (<80%, $+++P$ <0.001). In

contrast, there were no observed differences in SIRT3 mRNA levels between AL-WT and AL- AMPK^{-/-} mice (Fig. 3.1D).

In summary, CR did not increase gene expression of SIRT1, SIRT3 or SIRT6. In AL-fed mice, AMPK deficiency caused a modest decrease in SIRT1 and SIRT6, but did not affect SIRT3. Under CR, all sirtuins have decreased gene expression when AMPK is deficient. SIRT6, and to a lesser extent, SIRT1 are modestly decreased, while SIRT3 shows a significant marked decreased mRNA expression.

PGC- 1 α gene and protein expression and acetylation status

In skeletal muscle, PGC-1 α target genes for FA oxidation are induced by the deacetylase SIRT1 [24]. PGC-1 α controls the transcription of *SIRT3* to activate thermogenesis in brown adipose tissue [25] and to suppress the production of reactive oxygen species (ROS) in the mitochondria [26]. SIRT6 has not been reported to interact with PGC-1 α . Accordingly, and because SIRT6 changes were minimal in response to AMPK deficiency and CR, we speculated that PGC-1 α gene and protein expression would be altered in parallel with changes in SIRT1 and/or SIRT3 expression. Acetylation status was also assessed to verify whether SIRT1 activity altered in association with changes in SIRT1 gene expression. Skeletal-muscle PGC-1 α gene expression was similar in AL-WT mice and AL-AMPK^{-/-} mice. It has been reported that gene and protein expression of PGC-1 α in mouse skeletal muscle is enhanced by fasting [27] and, in the present experiments, PGC-1 α gene expression was substantially increased in CR-WT mice compared with AL-AMPK^{-/-} mice (>3-fold vs. AL-WT, ** P <0.01). The absence of AMPK led to ablation of CR-mediated increases in PGC-1 α gene expression (<300% vs. CR-WT, $\pm P$ <0.01) (Fig. 3.2A). The marked increase in PGC-1 α gene expression elicited by CR-WT mice was not, however, accompanied by any marked change in PGC-1 α protein.

PGC-1 α protein is deacetylated by SIRT1, and thus suppression of SIRT1 activity would be predicted to increase PGC-1 α protein acetylation. When deacetylated, PGC-1 α is activated and acts as a master regulator of mitochondrial biogenesis [28]. PGC-1 α protein was therefore immunoprecipitated and probed with anti-acetyl-lysine antibody to assess the acetylation level of PGC-1 α . Skeletal-muscle PGC-1 α acetylation was modestly (30%) but significantly ($*P$ <0.05) increased in AL-AMPK^{-/-}

mice compared with AL-WT mice, and substantially (0.5-fold, $*P<0.05$) increased in CR-WT animals compared with AL-WT mice (>0.5 -fold vs. AL-AMPK^{+/+}, $*P<0.05$). The absence of AMPK completely abrogated the CR-dependent increase in PGC-1 α protein acetylation ($<30\%$ vs. CR –WT, $\pm P<0.05$).

SIRT1 inhibition downregulates AMPK in starved L6 myoblasts

Ex527 is known to inhibit SIRT1 gene expression in different cell types [28, 29]. In agreement, we show here that SIRT1 mRNA levels were suppressed in starved L6 myoblasts, incubated with Ex527 ($\sim 100\%$ vs. control, $**P<0.01$; Fig. 3.3A). The impact of SIRT1 inhibition on AMPK expression and activity was analyzed in fed and starved cells. Although total-AMPK protein was unaffected by SIRT1 inhibition, a significant down regulation of phosphoAMPK-Thr¹⁷² protein expression (~ 0.5 -fold vs. control, $*P<0.05$) was observed in fed cells, suggesting that SIRT1 activity correlated positively with AMPK activity, contrasting with the inverse relationship between AMPK activation and Akt activation (Fig. 3.3B). In addition, similar findings were observed in serum-starved cells (Fig.3.3C) (Fig. 3.3B).

Loss of AMPK $\alpha 1/2$ attenuates insulin-mediated activation of Akt

It has been reported that, in vascular smooth muscle cells, tyrosine (Tyr) phosphorylation of IRS-1 is negatively regulated by serine⁷⁹⁴ phosphorylation of IRS-1 by activated AMPK [30]. We therefore postulated that lack of AMPK might affect Tyr phosphorylation of IRS-1 in skeletal muscle. Thus, we analyzed the Tyr phosphorylation level of skeletal-muscle IRS-1 through immunoprecipitation (Fig. 4A). In WT mice, total IRS-1 protein expression in skeletal muscle of AL mice was increased by insulin stimulation, but decreased in CR mice. AMPK deficiency did not exert any major effect on total-IRS-1 protein expression. AMPK deficiency did not markedly affect IRS tyrosine phosphorylation in AL-fed mice (without insulin treatment), but CR increased IRS tyrosine phosphorylation. CR and acute insulin treatment did not exert additive effects on IRS-1 tyrosine phosphorylation (Fig. 4A).

AL- and CR-WT mice exhibited remarkable increases in skeletal-muscle Akt Ser⁴⁷³ phosphorylation in response to insulin (>2 -fold vs. basal, $*P<0.05$; $\pm P<0.05$) (Fig. 4B and Fig. 4C). This demonstrated increased Akt activation since total-Akt protein was unchanged. The response of Akt-ser⁴⁷³ phosphorylation to insulin treatment was

greatly attenuated in AMPK^{-/-} mice (by 63% vs. AMPK^{+/+}, #*P*<0.05), (Fig 4B and Fig. 4C).

Discussion

We report here that CR-mediated improvement in whole body glucose tolerance is partly dependent on skeletal muscle AMPK activity. WT mice fed a CR diet displayed improved glucose tolerance, whilst this effect was ablated in the absence of skeletal muscle AMPK. These effects did not reflect differential effects in weight gain, since CR induced a significant body weight loss in WT and AMPK^{-/-} mice.

Rather, loss of CR-mediated improvements in glucose tolerance in AMPK^{-/-} mice likely reflect impaired skeletal muscle insulin signaling, since the beneficial effects of CR on insulin treated mice, reflected by tyrosine phosphorylation of IRS-1 were more reduced in AMPK^{-/-} mice compared to WT. AMPK phosphorylates and activates the insulin receptor, providing a direct link between AMPK and the insulin signaling pathway; this pathway promotes energy conservation and survival of muscle exposed to severe glucose deprivation [31]. In our study, it is clear that deficiency of AMPK α 1/2 impairs the decrease of IRS-1 phosphorylation induced by CR, upon the treatment with insulin. Given the known interaction between AMPK and mTORC-S6K1, these data suggest that a disrupted inactivation of mTORC-S6K1 signaling pathway contributes to the impaired insulin sensitizing effects of CR in AMPK^{-/-} mice treated with insulin [30]. AMPK-induced increase in skeletal muscle and liver insulin signaling pathway results in increased blood glucose uptake [32, 33]. In addition, AMPK activity also improves skeletal muscle and liver glycogen synthesis capacity by increased glycogen synthase activity [34, 35], enhancing blood glucose disposal, allowing faster glycaemia reduction.

In addition to increased skeletal muscle insulin sensitivity, we also observed reduced serum insulin levels 20 minutes-post IPGTT glucose infusion in CR-AMPK^{-/-} mice. This suggests that first-phase glucose-stimulated insulin secretion (GSIS) from pancreatic β -cells is partially suppressed when AMPK is absent from skeletal muscle. Therefore, these data highlight the intriguing possibility for a mechanism by which crosstalk between insulin-sensitive tissues and pancreatic islets might occur. In particular myokines produced by an AMPK-dependent process may be important for

regulation of β -cell insulin secretion in response to CR. This opens very interesting therapeutic perspectives. Further studies on β -cells of muscle specific AMPK^{-/-} mice must be done to better understand this pathway.

The importance of NAMPT in protecting the body against age-induced T2D has been reported [36]. This occurs in part through improved insulin secretion and glucose homeostasis [37], via increases in NAD⁺ levels and consequent SIRT1 activation. In this study we demonstrated that the AMPK inactivation in skeletal muscle is associated with changes in NAMPT expression and SIRT1 activity. Previous studies have shown that [27, 38] SIRT1 activity seems to be decreased when AMPK is lacking, as a result of decreased AMPK-mediated NAMPT expression, which suggest that SIRT1 and AMPK may be linked, [38]. However, the decrease of SIRT1 mRNA expression in skeletal muscle of AL-fed AMPK^{-/-} mice was no longer observed when mice were subjected to CR, suggesting that caloric restriction plays an important role in protecting skeletal muscle from the effects of decreased SIRT1 expression when AMPK is absent [39]. The compromised SIRT1 activity in AMPK^{-/-} mice abolished the SIRT1 dependent deacetylation of PGC-1 α in AL fed mice. This down regulation of PGC-1 α activity compromises its function on glucose and lipid metabolism [40]. Decreased PGC-1 α gene expression is a possible mechanism by which AMPK deficiency decreases insulin secretion and glucose uptake. Whilst some studies have reported increased SIRT1 mRNA and protein in response to CR [41], our study is consistent with previous reports that have shown SIRT1 gene and protein expression to be unaffected by CR in skeletal muscle and adipose tissue [42]. Interestingly, SIRT1 inhibition down regulates AMPK activity in L6 myoblasts, which suggests a possible feedback loop that might exist between these two proteins, where AMPK regulates SIRT1 activity through NAMPT concentrations and, on the other hand, SIRT1 regulates AMPK possibly through LKB1 deacetylation [43]. Xiao et al. found that 60% of SIRT6^(-/-) animals had very low levels of blood glucose and died shortly after weaning [22]. Feeding the mice with glucose-containing water increased blood glucose and rescued 83% of mutant mice, suggesting that the hypoglycemia is a major cause for the lethality. They showed that SIRT6 deficiency results in more abundant membrane association of GLUT1 and GLUT4, which enhances glucose uptake. The authors further demonstrated that SIRT6 negatively regulates Akt phosphorylation at Ser⁴⁷³ and Thr³⁰⁸ through inhibition of multiple upstream molecules, including insulin receptor, IRS1, and IRS2. The absence of SIRT6, consequently, enhances insulin

signaling and activation of Akt, leading to hypoglycemia. These data uncover an essential role of SIRT6 in modulating glucose metabolism through mediating insulin sensitivity [22]. Controversially, in our experiments, we found that reduced SIRT6 possibly due to reduced AMPK-mediated SIRT1 expression was associated with non-significant enhanced glucose plasma levels upon CR.

In summary, AMPK deficiency impairs the beneficial effects of CR is muscle insulin sensitivity, possibly because it is linked to decreased SIRT1 gene expression, which impairs insulin signaling pathway, culminating in reduced glucose uptake. Decreased AMPK in skeletal muscle might account for impaired GIIS signaling in pancreatic β -cells, reducing blood insulin content observed during GTT.

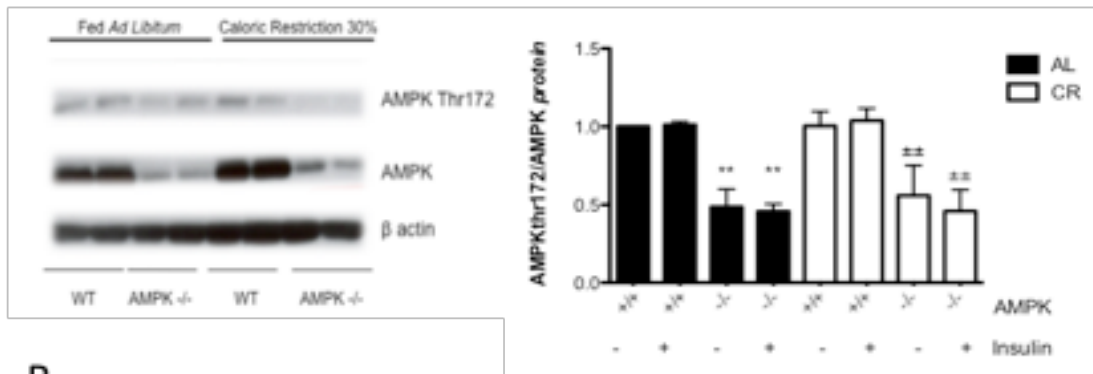
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Figure 1

A



B

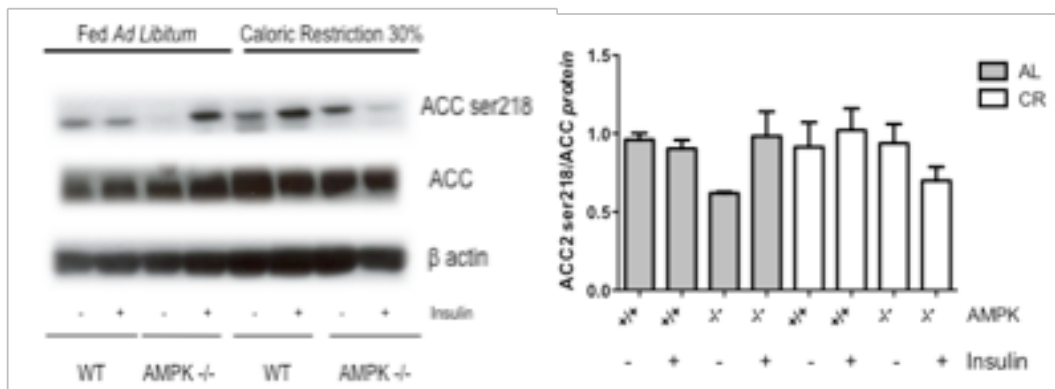


Figure 1. Representative western blot of total AMPK and phospho-AMPK-thr¹⁷² protein (MW: 63 kDa) (A); of total ACC and phospho-ACC-ser²¹⁸ protein (MW: 215 kDa) (B) and the control β -actin (MW: 45 kDa) are displayed. Data are means \pm SE. **P<0.005 vs. AL +/+, \pm P<0.005 vs. CR +/+, *P<0.05 vs. AL +/+, \pm P<0.05 vs. CR +/+. n= 4-5 per group. AL = ad libitum fed mice; CR = calorie restricted mice (30% caloric restriction); -/- = AMPK α 1/2 knockout mice; +/+ = WT mice.

Figure 2.

A

	WT		AMPK ^{-/-}	
	AL	CR	AL	CR
Initial weight (g)	18.52 ± 2.4	21.0 ± 2.3	17.33 ± 1.4	21.16 ± 2.7
Final weight (g)	22.34 ± 2.9	20.25 ± 1.0	21.33 ± 1.2	20.44 ± 2.6
Weight gain (g)	3.82 ± 1.3	-0.8 ± 2.3*** ###	4.0 ± 2.3	-0.72 ± 0.75*** ###

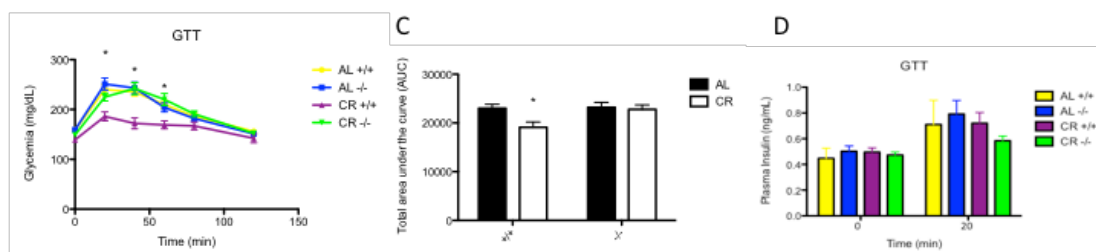
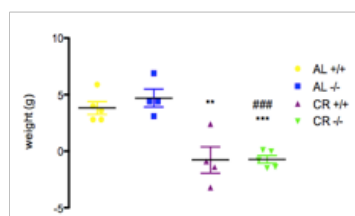


Figure 2. A Weight gained after 10 weeks of AL vs. CR treatment. Data are means \pm SE. *** $P < 0.001$ vs. AL +/+, ### $P < 0.001$ vs. AL -/-, $n = 4-5$ per group. -/- = AMPK $\alpha 1/2$ knockout mice. B, C & D: Glycaemia after intra-peritoneal administration of glucose (2g/kg of body weight) (B); graph showing AUC data (C); Plasma insulin levels measured after intra-peritoneal administration of glucose (2g/kg of body weight) (D). Data are means \pm SE ($n = 13-17$); * $P < 0.05$ CR +/+ vs. AL +/+, AL -/- and CR -/-. AL = ad libitum fed mice; CR = calorie restricted mice (30% caloric restriction); -/- = AMPK $\alpha 1/2$ knockout mice. +/+ = WT mice.

Figure 3.1

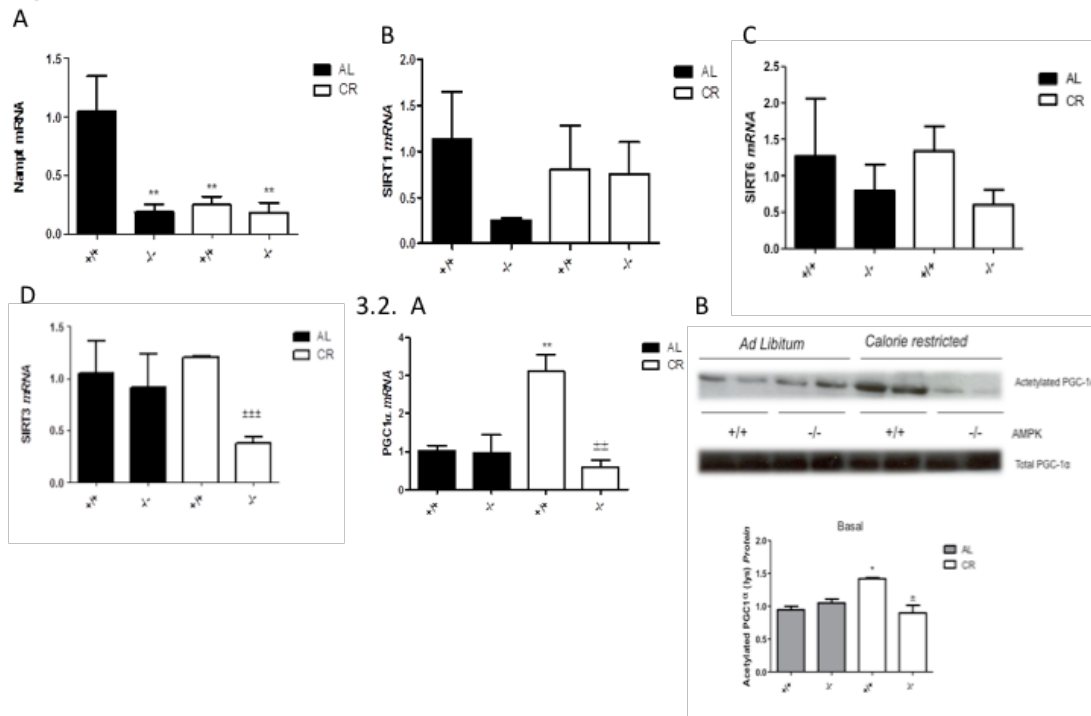


Figure 3.1. qRT-PCR showing NAMPT (A), SIRT1 (B) SIRT6 (C) and SIRT3 (D) mRNA expression in CR and AL mice (AMPK^{-/-} and WT) vs. 18S gene. Values are represented as means \pm SEM (n=4-6 per group). ** P <0.01 vs. +/+ AL mice; *** P <0.01 vs. +/+ CR. AL = ad libitum fed mice; CR = calorie restricted mice (30% caloric restriction); -/- = AMPK α 1/2 null mice. +/+ = WT mice.

Figure 3.2. A: qRT-PCR analysis showing PGC-1 α mRNA expression vs. 18S gene. B: Immunoprecipitation followed by western blotting analysis showing acetylation levels of PGC-1 α in lysine residues (MW: 130 kDa), compared to total PGC-1 α . Data are means \pm SE (n=4-6 per group), ** P <0.01 vs. +/+ AL, ± P <0.01 vs. +/+ CR, * P <0,05 vs. +/+ AL, ± P <0.05 vs. +/+ CR. AL = ad libitum fed mice; CR = calorie restricted mice (30% caloric restriction); -/- = AMPK α 1/2 null mice.

Figure 3.3

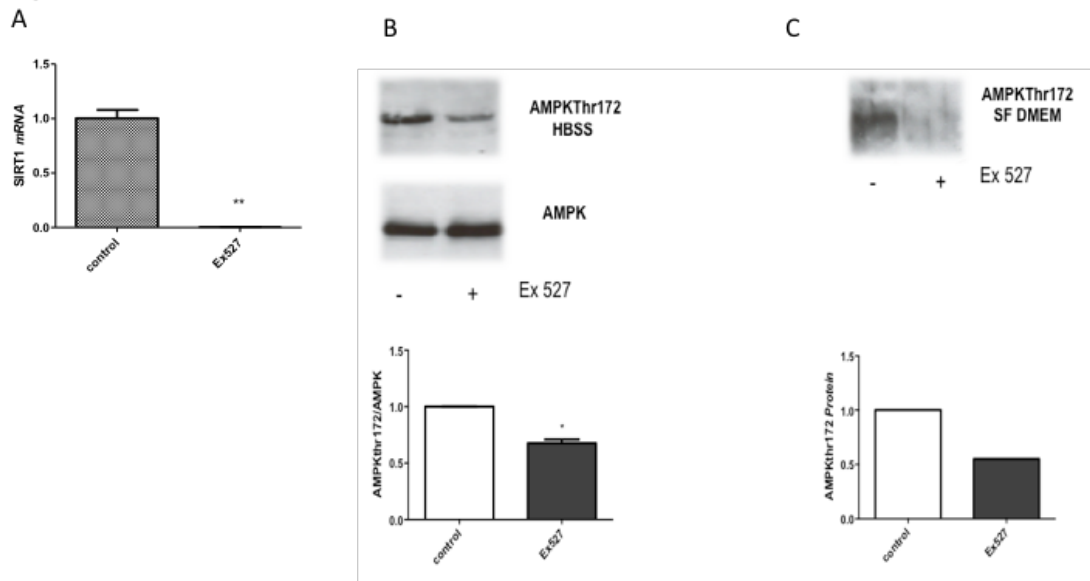


Figure 3.3: A: Changes in L6 SIRT1 mRNA level after culture with SIRT1 inhibitor, Ex527 (20 mg/ml of media), for 3.5 h. B & C: representative western blot of total AMPK and phospho-AMPK at Thr¹⁷² (MW: 63 kDa) changes in cells cultured with Ex527 (20 mg/ml of media) for 3.5h comparing to control cells [cells cultured in HBSS (serum-free, 1mM glucose) for 3.5h] (B) or comparing to control cells [cultured in serum-free DMEM (serum-free, 25 mM glucose) for 3.5h] (C). Total AMPK did not change between inhibit cells or control cells [cells cultured in HBSS (serum-free, 1mM glucose) for 3,5h] (B). Blots were repeated 3 times with qualitatively similar results. β -actin (MW: 45 kDa) was used to verify that the samples were equally loaded. Data are means \pm SE, * P <0.05 vs. control.

Figure 4

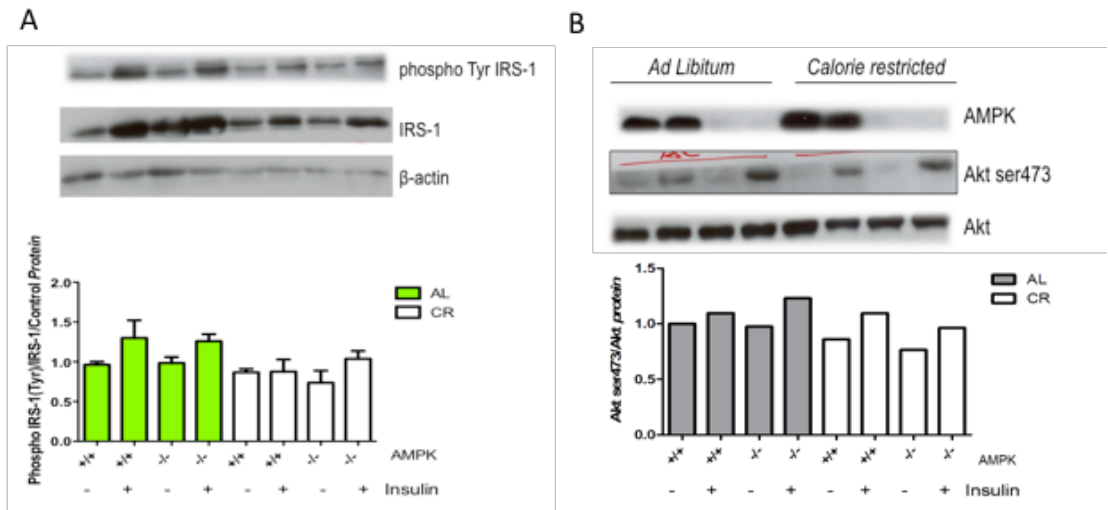


Figure 4. A: Immunoprecipitation followed by western blot analysis showing IRS tyrosine phosphorylation at tyrosine residues, compared to total IRS-1 protein expression (MW: 180 kDa). B: western blot analysis showing total-Akt and phospho-Akt-ser⁴⁷³ protein expression (MW: 56 kDa) in response to insulin administration (1 Unit/Kg of body weight administrated 5 min before sacrifice). B-actin (MW: 45 kDa) was used to verify that the samples were equally loaded. AL = ad libitum fed mice; CR = calorie restricted mice (30% caloric restriction); -/- = AMPK α 1/2 null mice. +/- = WT mice.