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Chronic AICAR treatment prevents metabolic changes in cardiomyocytes exposed to free fatty acids

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Abstract

The stimulation of glucose transport by metabolic stress is an important determinant of myocardial susceptibility to ischemia and reperfusion injury. Stimulation of glucose transport is markedly impaired in cardiomyocytes chronically exposed to excess free fatty acids (FFA), as occurs in vivo in type 2 diabetes. To determine whether chronic low-grade activation of AMP-activated kinase (AMPK) improves substrate metabolism in cardiomyocytes exposed to FFA, isolated cultured cardiomyocytes were exposed for 7 days to FFA ± the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Glucose transport and glycolysis were then measured during acute metabolic stress provoked by oligomycin. Chronic treatment with AICAR improved basal and oligomycin-stimulated glucose transport in FFA-exposed but not in control cardiomyocytes. Similarly, basal and oligomycin-stimulated glycolysis was reduced in FFA-exposed cardiomyocytes but restored by chronic AICAR treatment. Conversely, fatty acid oxidation was increased in FFA-exposed cardiomyocytes and reduced by chronic AICAR treatment. Chronic AICAR treatment induced in FFA-exposed cardiomyocytes the biogenesis of numerous lipid droplets. Curiously, whereas acute treatment of cardiomyocytes with AICAR increased phosphorylation of the AMPKα subunit on T^{172}, a classical marker of AMPK activation, chronic AICAR treatment almost completely obliterated T^{172} phosphorylation. However, phosphorylation of the AMPK target protein raptor on S^{792} was reduced in FFA-exposed cardiomyocytes but restored by AICAR treatment. In conclusion, chronic AICAR treatment induces a metabolic shift in FFA-exposed cardiomyocytes, characterized by improved glucose transport and glycolysis and redirection of fatty acids towards neutral storage. Such metabolic changes in vivo could protect the hearts of patients with type 2 diabetes against ischemia-reperfusion injury.

Keywords AMP-activated protein kinase (AMPK) · Glucose metabolism · Hyperlipidemia · Cardiomyocytes

Introduction

The heart is remarkably metabolically adaptable, being capable of using of wide variety of metabolic substrates to face the high ATP requirement due to incessant cardiac work. Among these substrates, glucose usually accounts for less than a quarter of the cardiac ATP production. Glucose is however important as a substrate from which energy can be extracted even during ischemia or hypoxia, from substrate-level phosphorylation in glycolysis. Stimulation of glucose uptake is thus a first line of defense for the myocardium undergoing ischemic stress. The importance of stimulation of glucose metabolism to withstand ischemic stress is illustrated by the poor recovery of GLUT4-null hearts [49] or hearts deficient in AMP-activated protein kinase (AMPK) [42], which are incapable of stimulation of glucose transport. Indeed, activation of the AMPK triggers the translocation of GLUT4 [41], stimulates glucose transport, and appears to mediate the effects of ischemia. Most importantly, impairment of AMPK-triggered stimulation of myocardial glucose uptake during ischemia and reperfusion is associated with reduced recuperation of contractile function and increased cardiac injury [42]. Stimulation of glucose transport is deficient in the myocardium of type II diabetes patients [22] as well as in animal models of type II diabetes [37]; this defect presumably contributes to the worse outcome of acute myocardial infarction in diabetic patients [1, 7] and to increased ischemia-reperfusion

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myocardial injury in animals [24]. Therefore, a pharmaceutical intervention that could improve glucose transport in the diabetic myocardium would open interesting therapeutic perspectives.

A hallmark of type II diabetes is the dyslipidemia, with increased circulating concentrations of free and lipoprotein-bound fatty acids, which is certainly one culprit of the dysregulation of myocardial glucose transport. Chronic exposure of cardiomyocytes to free fatty acids (FFA) [3, 47] or to triglyceride-rich lipoproteins [33] results in marked inhibition of basal, insulin-stimulated, and metabolic stress-stimulated glucose uptake. The mechanisms involved in the reduction of metabolic stress-stimulated glucose transport by FFA exposure involve inactivation of the focal adhesion kinase [51] and chronic activation of protein kinase C δ [52].

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) is an adenosine analog that is taken up by cells and phosphorylated into the AMP mimetics 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP) [9]; thus, AICAR activates AMPK independently of cellular metabolic stress, unlike biguanides. Therefore, AICAR-derived molecules have potential as treatment of type II diabetes [10, 16]. Bertrand et al. showed that AMPK activation with biguanides could improve glucose transport in cardiomyocytes that had been rendered resistant to insulin but not to metabolic stress [5]. In this study, we determined whether chronic treatment with low doses of AICAR could improve metabolic stress-stimulated glucose transport in cardiomyocytes exposed to FFA, in an effort to model in vitro the in vivo situation of type II diabetes treated with low-grade chronic AMPK activation.

Material and methods

Rat cardiomyocytes culture

Male Sprague Dawley rats (100–200 g) obtained from Janvier Labs (France) were euthanized by an intraperitoneal injection of pentobarbital (150 mg/kg) and hearts harvested under deep coma. Cardiomyocytes were isolated as previously described [2] by retrograde perfusion of the hearts with collagenase (type II; Worthington; 120 IU/ml) and hyaluronidase (1% *w*/*v*) [8, 14]. Cardiomyocytes were separated from non-myocyte cardiac cells by preplating the whole cell suspension for 90 min on untreated plastic, to which non-myocyte cells, but not cardiomyocytes, readily adhere. Cardiomyocytes were plated in M199 medium containing 5.5 mM glucose supplemented with 20 mM creatine, 100 μM cytosine-β-D-arabinofuranoside, 100 nM 9-*cis* retinoic acid, and 20% fetal calf serum (FCS). Dishes were previously coated with 0.1% gelatin for 4 h and incubated overnight with complete culture medium. For confocal microscopy, cardiomyocytes were plated on laminin-coated glass coverslips; for extracellular flux measurements, SeaHorse V7 plates were also coated with laminin. Free fatty acids (FFA) consisted of a 1:1 mix of palmitate (C16:0) and oleate (C18:1 n-9) bound to bovine serum albumin (BSA), at a final total concentration of 0.4 mM. FFA or AICAR were added at the time of plating. The culture medium was renewed every 2–3 days, and subsequent analyses were performed on day 7. At this time point, control cardiomyocytes display a well-differentiated phenotype with stable insulin responsiveness [39].

Metabolic measurements

Glucose transport was estimated by measuring 2-deoxyglucose (2-DG) uptake, as previously described [2, 31]. Briefly, cardiomyocytes were incubated in M199 containing 10 nM [2,6-^{3}H]-2-DG (ANAWA) (1–2 μCi/ml) and 5.5 mM cold glucose at 37 °C for 1 h, in the presence or absence of glucose transport agonists. Glucose transport agonists used were insulin (10^{-6} M) or oligomycin (10^{-6} M) to induce metabolic stress. 2-DG uptake was stopped by three washes with ice-cold PBS before lysis in 1 ml 0.1 M NaOH. Two 20-μl aliquots were taken for protein content determination and the remaining NaOH lysate assayed for radioactivity in a TriCARB 1900 TR liquid scintillation analyzer (Packard).

Glycolytic rates were estimated from the detritiation of [5-^{3}H]-glucose [48]. Cardiomyocytes were incubated in M199 containing 10 nM [5-^{3}H]-glucose (ANAWA) (1–2 μCi/ml) and 5.5 mM cold glucose at 37 °C for 1 h, in the presence or absence of glucose transport agonists. At the end of the incubation period, the incubation medium was retrieved, immediately diluted three times with ice-cold water, and ^3H_2O was separated from [5-^{3}H]-glucose by anion exchange chromatography in Dowex 1 × 4 $B_4O_7^{2-}$. ^3H_2O eluted from the Dowex column was counted by liquid scintillation.

Palmitate oxidation was estimated based on the rate of transfer of ^{3}H from [9,10-^{3}H]palmitate to ^3H_2O [28]. For measurement of palmitate oxidation, cardiomyocytes were incubated for 60 min in medium containing palmitate (0.05 mM), oleate (0.05 mM), and 1 μCi/ml [9,10-^{3}H]palmitate complexed to bovine serum albumin (0.2 mM). At the end of the incubation period, the incubation medium was retrieved, immediately diluted three times with ice-cold water, and ^3H_2O was separated from [9,10-^{3}H]palmitate by anion exchange chromatography in Dowex 1 × 4 OH^-. ^3H_2O eluted from the Dowex column was counted by liquid scintillation. Cardiomyocytes were washed twice with ice-cold PBS, dissolved in 0.1 M NaOH. Twenty-microliter aliquots were taken for protein content determination and the remaining NaOH lysate assayed for radioactivity by liquid scintillation. Palmitate uptake was estimated from the sum of ^{3}H label transferred to ^3H_2O and ^{3}H label remaining in the cardiomyocytes.

Oxygen consumption rates were measured in a SeaHorse XFe24 extracellular flux analyzer, following the protocol developed by Readnower et al. [38]. The medium used was unbuffered DMEM (Sigma no. D5030) containing 1 mM pyruvate, 4 mM L-glutamine, and 5 mM glucose.

Preparation of cytosol and membrane fractions

Cytosol and membrane fractions of cultured cardiomyocytes were obtained as previously described [53]. Cardiomyocytes were washed in ice-cold PBS and scraped in buffer A containing 20 mM Tris-HCl pH 7.5, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 2 mM dithiothreitol, and Halt protease and phosphatase inhibitor cocktail. The suspension was sonicated with four 5-s bursts on ice and then centrifuged at 1500×*g* for 10 min. The supernatant was then ultracentrifuged at 100,000×*g* for 45 min at 4 °C. The pellet containing the membrane fraction was solubilized in buffer A containing 1% Triton X-100, sonicated, and centrifuged at 15,000×*g* for 15 min at 4 °C to retain the supernatant.

Immunoblot analysis

Following stimulation with insulin or oligomycin, incubations were terminated by three washes in ice-cold PBS before solubilizing cells in 200 μl lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Igepal CA 630, Halt protease, and phosphatase inhibitor Cocktail (Pierce, Thermo Scientific). Proteins (30 μg) from each sample were separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Primary antibodies used are listed in Table 1. Secondary anti-mouse IgG (115-035-146) and anti-rabbit IgG (111-035-003) HRP-conjugated antibodies were from Jackson ImmunoResearch. Densitometric analysis of chemiluminescent signals captured with a LAS-4000 Luminescent Image Analyzer (Fujifilm) was performed using the ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij). For each separate membrane, the data were normalized such that the highest intensity, regardless of treatment, was equal to one.

Confocal microscopy

For confocal fluorescent microscopy, cardiomyocytes cultured on laminin-coated glass coverslips were washed with ice-cold PBS and fixed with 4 mM paraformaldehyde in PBS for 20 min at room temperature. Fixation was quenched with 200 mM glycine in PBS and cardiomyocytes permeabilized with 0.3% Triton X-100 in PBS for 3 min. Non-specific dye or antibody binding was reduced by preincubation with 3% BSA and 0.1% Tween-20 in PBS. Neutral lipids were stained with 2.5 μg/ml Bodipy 493/503 for 30 min [19]; F-actin counterstaining was obtained with AlexaFluor 543-labeled phalloidin (2 U/ml).

For cytoskeleton imaging, fixed and permeabilized cardiomyocytes were incubated overnight at 4 °C with a mouse α-actinin antibody and a rabbit α-tubulin antibody (see Table 1), followed by secondary antibodies AlexaFluor

Table 1 Primary antibodies used in this study

Antigen	Company	Reference no.	Application
Acetyl-CoA carboxylase	Cell Signaling Technology	3676	Western blot
Acetyl-CoA carboxylase, phosphoS^{79}	Cell Signaling Technology	11,818	Western blot
AMPKα	Cell Signaling Technology	2532	Western blot
AMPKα, phosphoS^{485}	Cell Signaling Technology	4184	Western blot
AMPKα, phosphoT^{172}	Cell Signaling Technology	2531	Western blot
AMPKα, phosphoT^{172}	Cell Signaling Technology	2535	Western blot
AMPKα, phosphoT^{172}	Bioss Antibodies	bs-4002R-A555	Immunofluorescence
Connexin 43	Cell Signaling Technology	3512	Western blot
GLUT1	Abcam	ab652	Western blot
GLUT4	Abcam	ab654	Western blot
PKCα	BD Transduction Labs	610,107	Western blot
PKCδ	BD Transduction Labs	610,397	Western blot
PKCε	BD Transduction Labs	610,085	Western blot
PKCζ, phosphoT^{410}	Cell Signaling Technology	2060	Western blot
Raptor	Cell Signaling Technology	2280	Western blot
Raptor, phosphoS^{792}	Cell Signaling Technology	2083	Western blot
α-Actinin	Sigma-Aldrich	A7811	Western blot, immunofluorescence
α-Tubulin	Abcam	ab15246	Immunofluorescence

488-labeled F(ab′)2 goat anti-rabbit IgG (Invitrogen A11070) and AlexaFluor 633-labeled F(ab′)2 goat anti-mouse IgG (Invitrogen A21053); F-actin counterstaining was obtained with AlexaFluor 543-labeled phalloidin (2 U/ml).

Following washes with PBS and H_2O, coverslips were mounted on glass slides with ProLong Diamond antifade containing DAPI for DNA staining. Cardiomyocytes were examined with a Leica SP5 (Fig. 5) or a Zeiss LSM800 (Fig. 8) confocal microscope, using a ×63 oil immersion objective. One-micrometer-thick confocal slices were acquired throughout the thickness of the cardiomyocytes and Z-stack projections obtained with the ImageJ software in maximum intensity projection mode. Image luminosity and contrast were digitally enhanced, taking care of applying the same linear adjustments to images from different experimental groups [40].

Statistics

Data are presented as mean ± SD obtained from replicated experiments. Data were compared by one-way or two-way ANOVA (Prism 7, GraphPad Software) followed by post hoc testing for false discovery rates by the method of Benjamini and Yekutieli [4]. ANOVA results are indicated with p values in the text of the "Results" section. Post hoc testing indicated a positive discovery when the false discovery rate q was < 0.05. Throughout the article, the following symbols are used for positive discoveries (q values): * indicates a significant effect of insulin or oligomycin stimulation as compared with unstimulated cardiomyocytes having received the same chronic treatment; # indicates a significant effect of chronic FFA exposure, as compared with cardiomyocytes not exposed to FFA undergoing the same acute stimulation; and § indicates a significant effect of chronic AICAR exposure (but with the same exposure—or absence thereof—to FFA), as compared with cardiomyocytes not exposed to AICAR undergoing the same acute stimulation.

Results

Effects of chronic AICAR treatment in control and FFA-exposed cardiomyocytes on glucose metabolism

In cultured primary cardiomyocytes, both insulin and oligomycin acutely stimulated glucose uptake, 3- and 3.4-fold, respectively (Fig. 1a). As we previously reported [3, 51, 52], chronic (7 days) exposure of primary cardiomyocytes to 0.4 mM FFA markedly reduced basal and stimulated glucose uptake. Remarkably, chronic treatment of control cardiomyocytes with 0.2 mM AICAR abolished insulin-

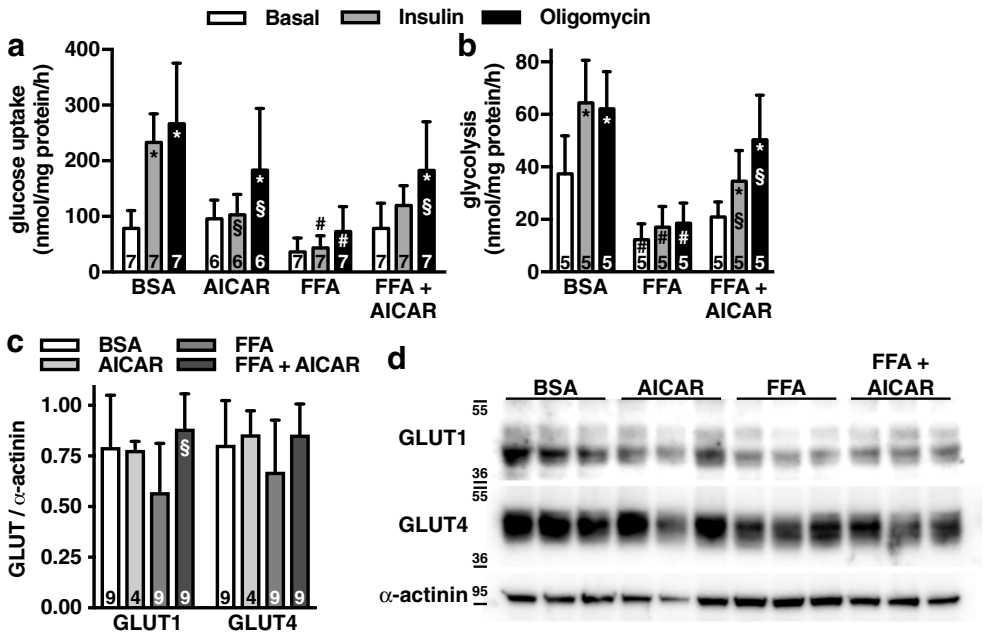


Fig. 1 Chronic AICAR treatment improves glucose metabolism in cardiomyocytes exposed to FFA. Cardiomyocytes were cultured for 7 days in the presence (FFA) or absence (BSA) of 0.4 mM free fatty acids concomitantly with 0.2 mM AICAR or its vehicle. Glucose uptake (**a**) or glycolysis (**b**) was measured during 1 h in the presence of 10^{-6} M insulin or 10^{-6} M oligomycin following chronic treatment with FFA ± AICAR. **c** Expression of the glucose transporters GLUT1 and GLUT4 relative to that of α-actinin, expressed in arbitrary units. **d** Representative Western blots of glucose transporters GLUT1 and GLUT4 expression. Results are mean ± SD of the number of separate experiments indicated at the foot of each column. * : $q < 0.05$ for the effect of insulin or oligomycin. # : $q < 0.05$ for the effect of FFA. § : $q < 0.05$ for the effect of AICAR

stimulated glucose uptake and reduced oligomycin-stimulated glucose uptake. On the other hand, AICAR treatment significantly improved oligomycin-stimulated glucose uptake in FFA-exposed cardiomyocytes, with non-significant trends towards amelioration of basal and insulin-stimulated glucose uptake; thus, chronic AICAR treatment essentially nullified the effects of FFA on oligomycin-stimulated glucose uptake in cardiomyocytes.

The variations of glycolytic activity were parallel to those on glucose uptake (Fig. 1b), although with glycolytic rates consistently lower than the rates of glucose uptake. Both insulin and oligomycin stimulated glycolysis in control cardiomyocytes, albeit to a relatively smaller extent than glucose uptake (1.7-fold). FFA reduced basal and stimulated glycolysis, while concomitant treatment with AICAR improved insulin-stimulated and almost completely restored oligomycin-stimulated glycolysis.

Glucose uptake by cardiomyocytes is mediated by two glucose transporters of the GLUT family, GLUT1 and GLUT4. We previously showed using the GLUT4-specific inhibitor indinavir that about 40% of basal glucose uptake and 80% of insulin- or oligomycin-stimulated glucose uptake were GLUT4-mediated in control cultured cardiomyocytes in our model [52]. The expression of neither GLUT1 nor GLUT4 was significantly affected by the culture conditions, with the exception of AICAR significantly restoring expression of GLUT1, which was blunted by exposure to FFA ($q = 0.074$) (Fig. 1c, d).

Effects of chronic AICAR treatment in control and FFA-exposed cardiomyocytes on AMPK signaling

As both AICAR, chronically in these settings, and oligomycin, acutely, are expected to act through activation of the AMPK complex, we examined AMPK signaling in cardiomyocytes chronically exposed either AICAR, FFA, or both. Phosphorylation of the AMPKα subunit on T^{172} is classically taken as indicative of AMPK activation [18]; indeed, in control cardiomyocytes, inhibition of mitochondrial ATP synthase with oligomycin resulted in robust phosphorylation of T^{172}-AMPKα (Fig. 2a). A similar effect was observed in FFA-exposed cardiomyocytes, without any difference in T^{172}-AMPKα phosphorylation brought about by FFA exposure. Quite surprisingly though, phosphoT^{172}-AMPKα was much reduced, to the point of being almost undetectable in some experiments, in cardiomyocytes exposed to AICAR, be it in the presence or not of FFA, even after stimulation with oligomycin. This was not caused by downregulation of global AMPK expression, as expression of the AMPKα subunit was slightly but significantly increased by AICAR treatment, both in the presence ($q = 0.0152$) or absence of FFA ($q = 0.0004$).

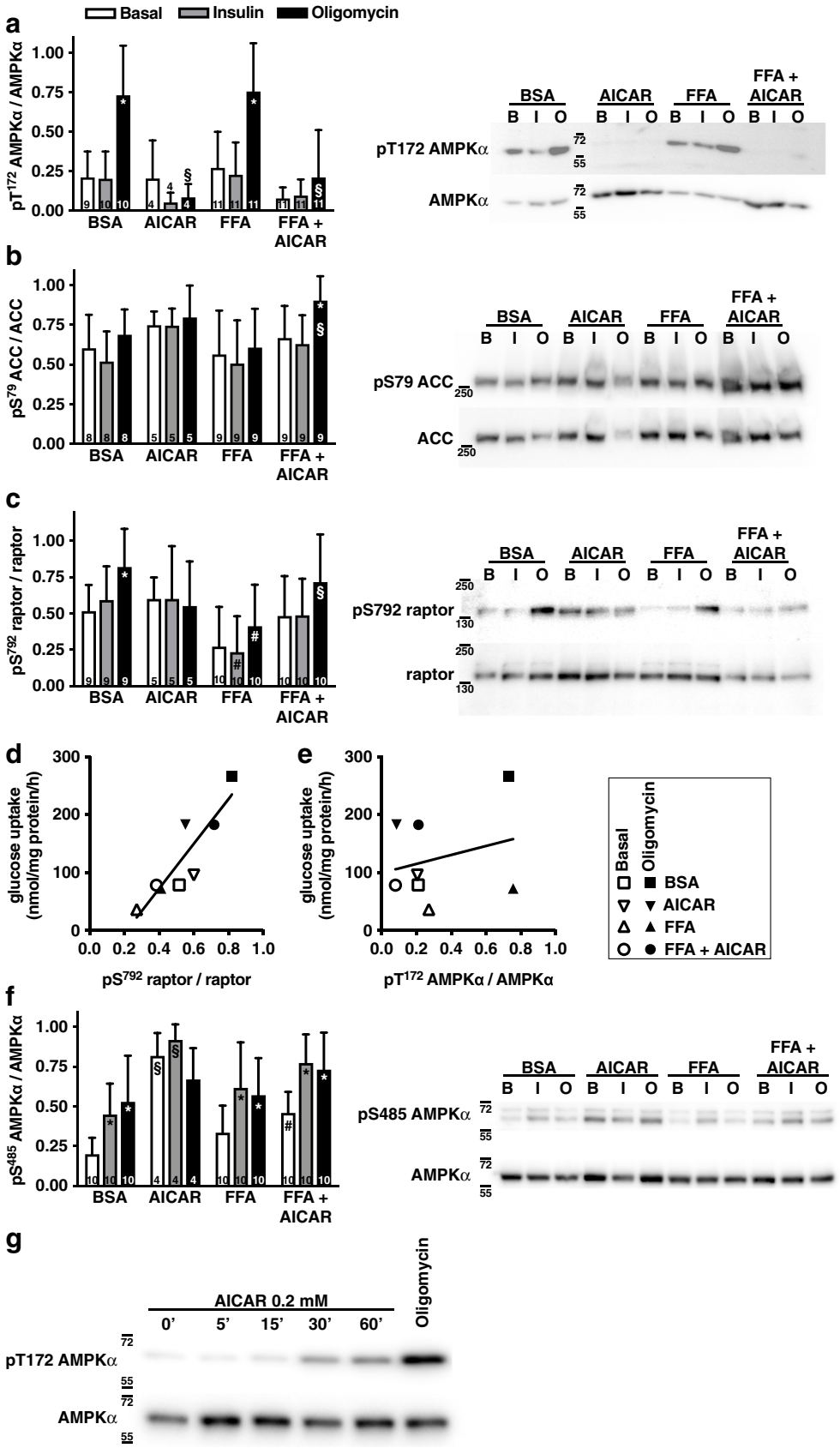
To further evaluate the activity of the AMPK complex, we assessed the phosphorylation of two of its known substrates, acetyl-CoA carboxylase [34] (ACC; Fig. 2b) and raptor [17] (Fig. 2c). There was a trend across all four culture conditions for phosphoS^{79}-ACC to be higher in oligomycin-stimulated than in insulin-stimulated cardiomyocytes ($p = 0.0064$ by two-way ANOVA). Chronic AICAR treatment, either in control ($p = 0.008$) or in FFA-exposed cardiomyocytes ($p = 0.0069$), increased phosphorylation of S^{79}-ACC, consistent with a possible activation of AMPK by AICAR. FFA had no effect on phosphorylation of S^{79}-ACC. Neither AICAR nor FFA influenced global expression of ACC. These results are consistent with oligomycin- or AICAR-activated AMPK phosphorylating ACC [34] and insulin promoting dephosphorylation of ACC [55]; the sensitivity of S^{79}-ACC phosphorylation as a readout of AMPK activity in cultured cardiomyocytes appeared however to be rather low.

Acute stimulation with oligomycin induced the phosphorylation of raptor on S^{792} in control cardiomyocytes ($q = 0.019$); curiously, this effect was lost in cardiomyocytes exposed to AICAR only. In cardiomyocytes exposed to FFA, phosphoS^{792}-raptor was reduced overall ($p = 0.0001$), but a trend towards an increase with oligomycin remained. In cardiomyocytes exposed to both FFA and AICAR, the control pattern of S^{792} phosphorylation was restored ($p = 0.0007$ vs. FFA only). Neither AICAR nor FFA influenced global expression of raptor.

Thus, phosphoS^{792}-raptor taken as a readout of AMPK activity correlated better with glucose uptake in the various culture and stimulation conditions ($r^2 = 0.797$; $p = 0.0028$; Fig. 2d) than phosphoT^{172}-AMPKα did ($r^2 = 0.072$; $p = 0.52$; Fig. 2e); data with insulin stimulation were omitted from this analysis as they are not pertaining to AMPK-mediated stimulation of glucose uptake.

Because of the apparent dissociation between T^{172}-AMPKα phosphorylation and glucose uptake in conditions of metabolic stress after exposure to FFA ± AICAR, we considered whether phosphorylation of an inhibitory site on AMPKα [21] could explain some of these discrepancies (Fig. 2f). As previously described, insulin induced the phosphorylation of S^{485}-AMPKα and so did oligomycin-induced metabolic stress. Notably however, these effects were not observed in cardiomyocytes exposed to AICAR alone. The addition of AICAR significantly increased phosphorylation of S^{485}-AMPKα, both in the presence ($p = 0.0097$) or absence ($p < 0.001$) of FFA. Culture of cardiomyocytes in the presence of FFA tended to increase phosphorylation of S^{485}-AMPKα ($p = 0.051$). Therefore, S^{485}-AMPKα phosphorylation does not correlate with glucose uptake either.

In cultured cardiomyocytes, we observed that 1 h of incubation with 0.2 mM AICAR resulted in a slight increase in T^{172}-AMPKα phosphorylation (207 ± 153% of basal; mean ± SD, $n = 4$, $q = 0.24$) of AMPK, much lesser than that achieved by oligomycin exposure (732 ± 305% of basal; mean ± SD,


a
Basal
Insulin
Oligomycin
pT172 AMPKα / AMPKα
BSA
AICAR
FFA
FFA + AICAR
pT172 AMPKα
AMPKα
b
pS79 ACC / ACC
pS79 ACC
ACC
c
pS792 raptor / raptor
pS792 raptor
raptor
d
glucose uptake (nmol/mg protein/h)
pS792 raptor / raptor
e
pT172 AMPKα / AMPKα
Basal
Oligomycin
f
pS485 AMPKα / AMPKα
pS485 AMPKα
AMPKα
g
AICAR 0.2 mM
0'
5'
15'
30'
60'
Oligomycin
pT172 AMPKα
AMPKα

◀ **Fig. 2** AMPK signaling in cardiomyocytes exposed to FFA and AICAR. **a**–**e** Cardiomyocytes were cultured for 7 days in the presence (FFA) or absence (BSA) of 0.4 mM free fatty acids concomitantly with 0.2 mM AICAR or its vehicle. Cardiomyocytes were then stimulated for 10 min with 10^{-6} M insulin or for 20 min with 10^{-6} M oligomycin. Left panels: quantitative analysis of the phosphorylation states of AMPKα (on T^{172}, **a;** on S^{485}, **f**); acetyl-CoA carboxylase (ACC; on S^{79}, **b**) and raptor (on S^{792}, **c**). Results are mean ± SD of the number of separate experiments indicated at the foot of each column. * : $q < 0.05$ for the effect of insulin or oligomycin. # : $q < 0.05$ for the effect of FFA. § : $q < 0.05$ for the effect of AICAR. Right panels: representative Western blots for each phosphorylation site. **d**, **e** Correlation between phosphorylation of raptor (phosphoS^{792}) or AMPK (phosphoT^{172}) and glucose uptake. Squares, BSA; triangles, FFA; circles, FFA + AICAR; white symbols, no stimulation; black symbols, oligomycin stimulation. Insulin data points were omitted from this analysis. **g** AMPK activation in freshly isolated cardiomyocytes stimulated with 0.2 mM AICAR or 10^{-6} M oligomycin for 5–60 min; Western blots representative of four separate experiments

$n = 4$, $q = 0.0015$; Fig. 2g); phosphorylation of raptor on S^{792} was not increased (data not shown).

To determine whether stimulation of glucose uptake by oligomycin under chronic FFA + AICAR was actually AMPK-mediated, we measured glucose uptake and raptor phosphorylation in the presence of the AMPK inhibitor compound C (CC) [56]. CC present acutely during incubation with oligomycin prevented the stimulation of glucose uptake both in control cardiomyocytes, as expected, and in cardiomyocytes exposed to chronic FFA + AICAR (Fig. 3a). Similarly, the phosphorylation of raptor on S^{792} was markedly diminished in the presence of CC (Fig. 3b).

Differential localization of activated AMPK, thereby targeting different substrates, could also potentially explain the discrepancies between phosphoT^{172}-AMPKα, phosphorylation of AMPK substrates, and glucose uptake. To address this question, we first performed immunofluorescence studies of phosphoT^{172}-AMPKα in ex vivo cardiomyocytes to validate the antibody used for immunolabeling. As shown in Fig. 4a, stimulation of ex vivo cardiomyocytes with 1 μM oligomycin for 30′ resulted in increased immunolabeling of phosphoT^{172}-AMPKα, with a distinct striated and nuclear distribution, thus matching findings by other authors who studied the subcellular distribution of AMPKγ in cardiomyocytes [36]. In cultured control cardiomyocytes stimulated with oligomycin, this striated and nuclear pattern of phosphoT^{172}-AMPKα subcellular distribution was maintained, notwithstanding the extensive cytoskeletal remodeling taking place during the 7-day culture period (Fig. 4b). In contrast in both FFA- and FFA + AICAR-exposed cardiomyocytes, the cytoplasmic distribution of phosphoT^{172}-AMPKα appeared to be more diffuse and less intense, perhaps in relation with the disorganization of the cytoskeleton taking place in these culture conditions (see Fig. 8); nuclear distribution of phosphoT^{172}-AMPKα remained prominent.

Effects of chronic AICAR treatment in FFA-exposed cardiomyocytes on lipid metabolism

In previous works, we obtained indirect evidence that some by-product(s) of fatty acid oxidation (FAO) could play a role in the reduction of oligomycin-stimulated glucose uptake [3], whereas funneling of fatty acids to triglycerides was associated with preservation of oligomycin-stimulated glucose uptake [52]. We therefore examined fatty acid metabolism in

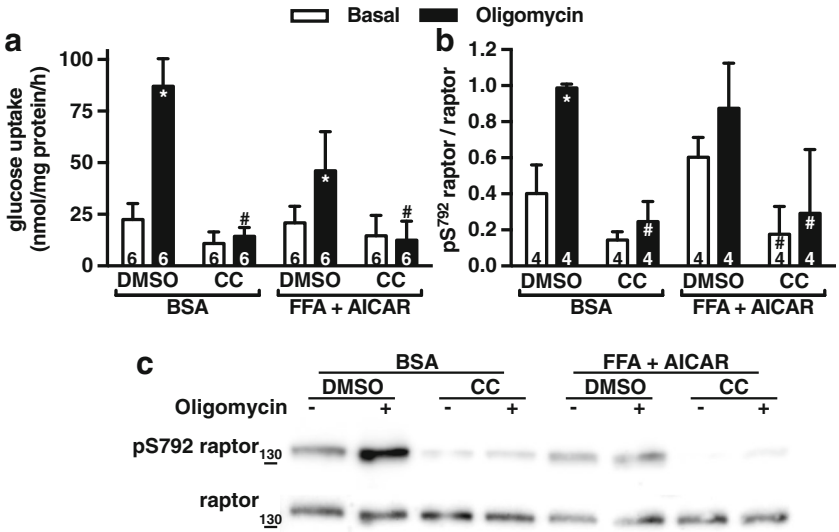


Fig. 3 AMPK-dependence of glucose uptake. Cardiomyocytes were cultured for 7 days in medium containing BSA or 0.4 mM free fatty acids ± 0.2 mM AICAR. Glucose uptake (**a**) or phosphorylation of raptor on S^{792} (**b**) was measured during 1 h in the presence or absence of 10^{-6} M oligomycin and 10^{-5} M compound C (CC) following chronic treatment with BSA or FFA + AICAR. DMSO is the vehicle of CC. Results are mean ± SD of the number of separate experiments indicated at the foot of each column. * : $q < 0.05$ for the effect of oligomycin. # : $q < 0.05$ for the effect of CC. **c** Representative Western blots of phosphorylated and total raptor

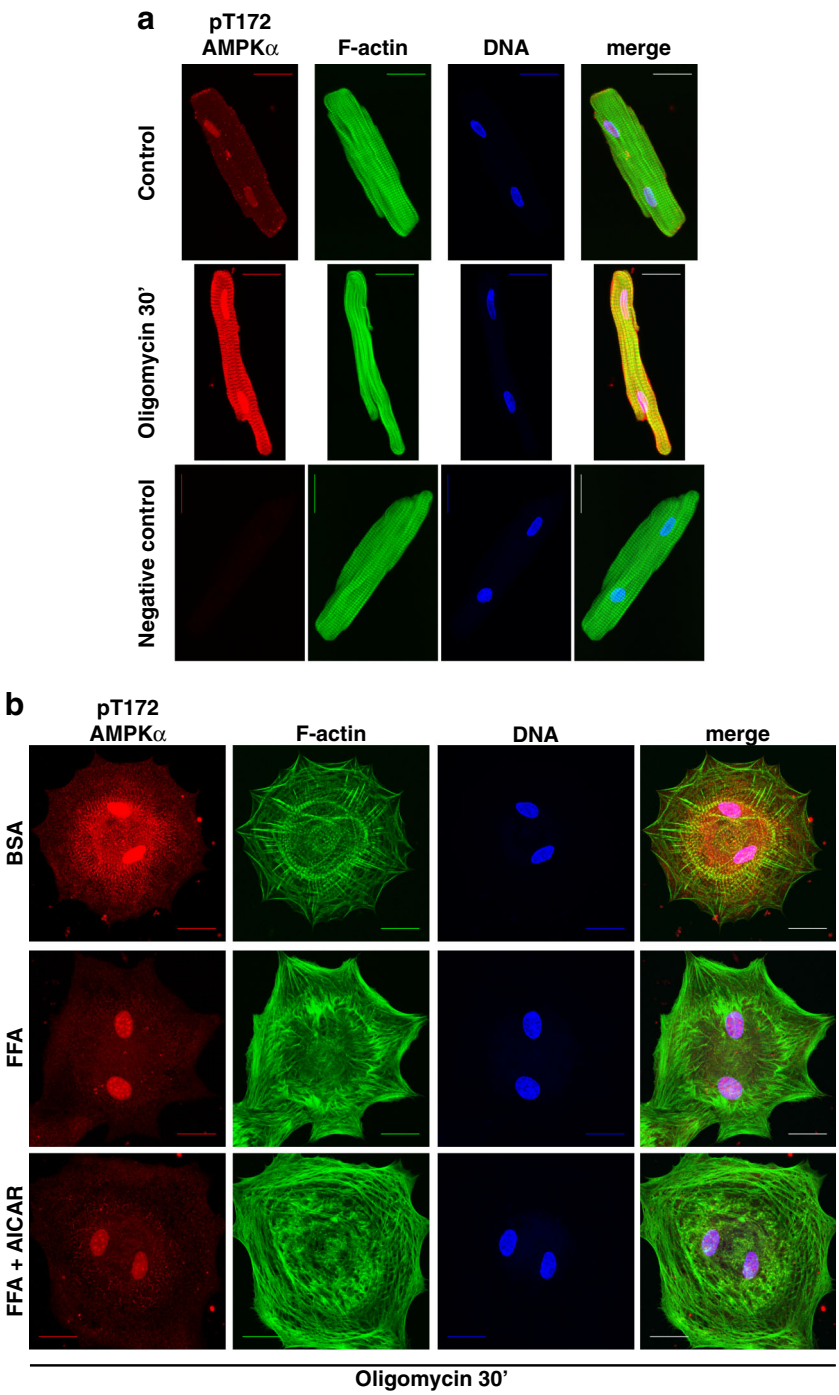


Fig. 4 Active AMPK localization in cardiomyocytes exposed to FFA and AICAR. **a** Freshly isolated cardiomyocytes were left quiescent or challenged for 30 min with 10^{-6} M oligomycin, fixed, permeabilized, and stained with an AlexaFluor555-conjugated phosphoT^{172} AMPKα antibody (red); counterstaining for F-actin (green) was obtained with phalloidin and for DNA (blue) with DAPI. The phosphoT^{172} AMPKα antibody was omitted for the negative control. **b** Cardiomyocytes were cultured for 7 days in medium containing BSA or 0.4 mM free fatty acids (FFA) ± 0.2 mM AICAR. Cardiomyocytes were then stimulated for 30 min with 10^{-6} M oligomycin and stained as above. Scale bar 20 μM

cardiomyocytes exposed to FFA ± AICAR (Fig. 5). Palmitate uptake and oxidation were both increased in cardiomyocytes exposed to FFA, as was to be expected, as fatty acids stimulate expression of proteins of their own import and oxidation [50]. Addition of AICAR to FFA during the 7-day culture period did not alter palmitate uptake but reduced palmitate oxidation down to control levels. Thus, fractional palmitate oxidation, i.e., the fraction of palmitate taken up that is oxidized, was significantly increased in cardiomyocytes exposed to FFA only and reduced in cardiomyocytes exposed to both FFA and AICAR, suggesting a different intracellular fate of fatty acids taken up under AICAR. One possibility was that fatty acids were redirected towards storage in neutral lipids, as occurs in a similar situation of restoration of glucose transport by chronic exposure to a phorbol ester [52]. Indeed, we observed that exposure of cardiomyocytes to FFA + AICAR induced a marked biogenesis of lipid droplets (Fig. 5d). Both the area occupied by lipid droplets (Fig. 5e) and the number of lipid

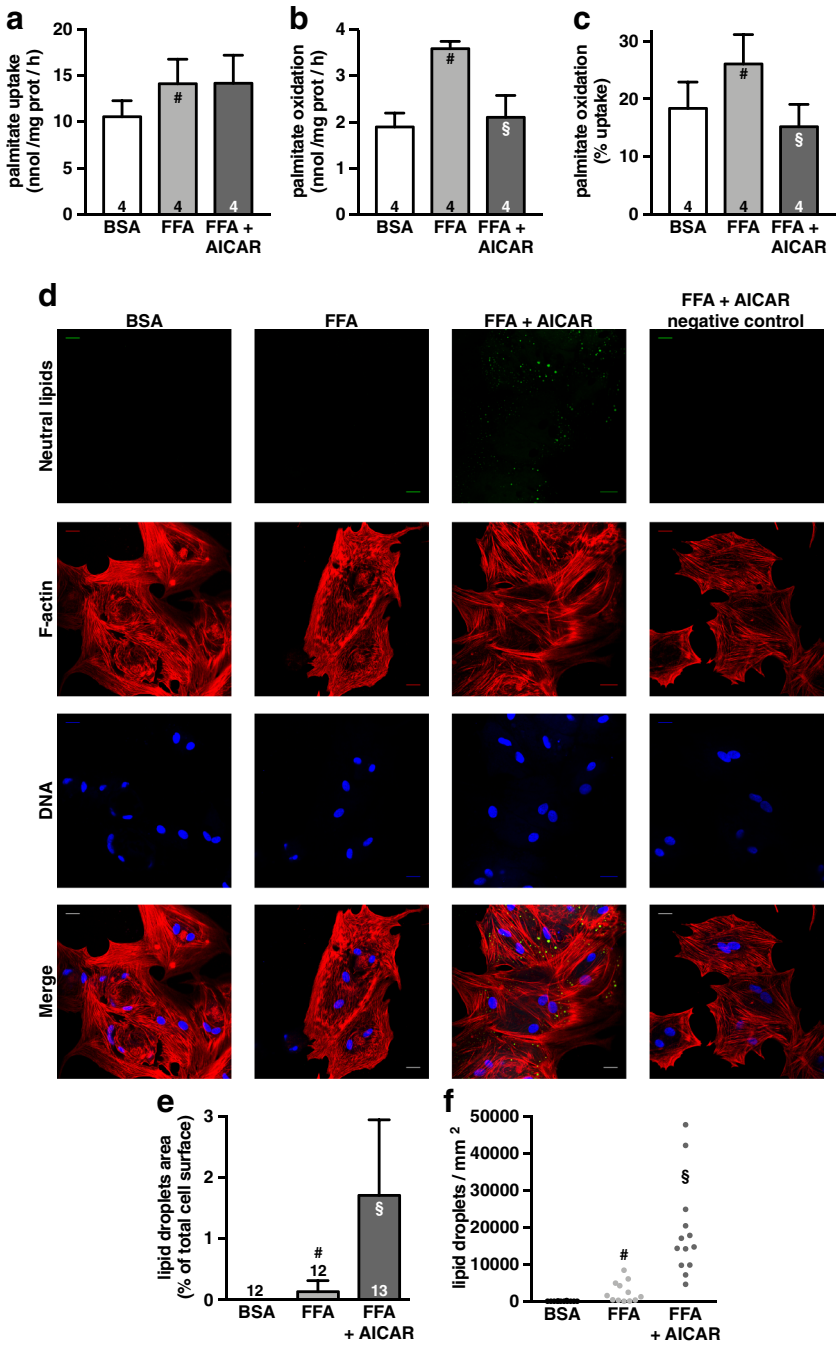


Fig. 5 Effect of chronic AICAR on fatty acid metabolism. Cardiomyocytes were cultured for 7 days in medium containing BSA or 0.4 mM free fatty acids (FFA) ± 0.2 mM AICAR. **a** Palmitate uptake was estimated from the sum of palmitate oxidation and ^{3}H label remaining in the cardiomyocytes. **b** Palmitate oxidation was estimated from the rate of transfer of ^{3}H from [9,10-^{3}H]palmitate to $^{3}H_2O$. **c** Palmitate oxidation expressed as a percentage of palmitate uptake. **d** Fluorescent imaging of lipid droplets. Green, neutral lipids; red, F-actin; blue, DNA. Scale bar 20 μm. In the negative control, the neutral lipid label (Bodipy 493/503) has been omitted while staining cardiomyocytes with numerous lipid droplets. Images are from a representative experiment. **e** Percentage of cell surface area labeled as neutral lipids. Results are mean ± SD. **f** Number of lipid droplets per square millimeter of cell surface area. For both **e** and **f**, 12 microscopic frames per condition over 3 separate experiments were analyzed. # : $q < 0.05$ for the effect of FFA. § : $q < 0.05$ for the effect of AICAR

droplets by square millimeter of cell area (Fig. 5f) were significantly increased in cardiomyocytes exposed to FFA + AICAR as compared to FFA only, where very few lipid droplets were visible altogether.

Chronic AICAR exposure could reduce mitochondrial activity, which would then explain the observed decrease in fatty acid oxidation. If this were the case, the increase in glucose uptake could be simply due to the inhibition of both glucose and fatty acid oxidation, anaerobic glycolysis remaining the sole way to produce energy. To evaluate this possibility, we assessed basal and maximal mitochondrial activity by monitoring O_2 consumption rates (OCR) in the presence of 1 mM pyruvate and 5 mM glucose. Mitochondrial OCR were obtained by subtracting OCR measured in the presence of 10^{-5} M antimycin A [38]. Both the basal and the maximal OCR, induced by the mitochondrial uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), were reduced in cardiomyocytes chronically exposed to FFA (Fig. 6); AICAR slightly but not significantly improved basal and

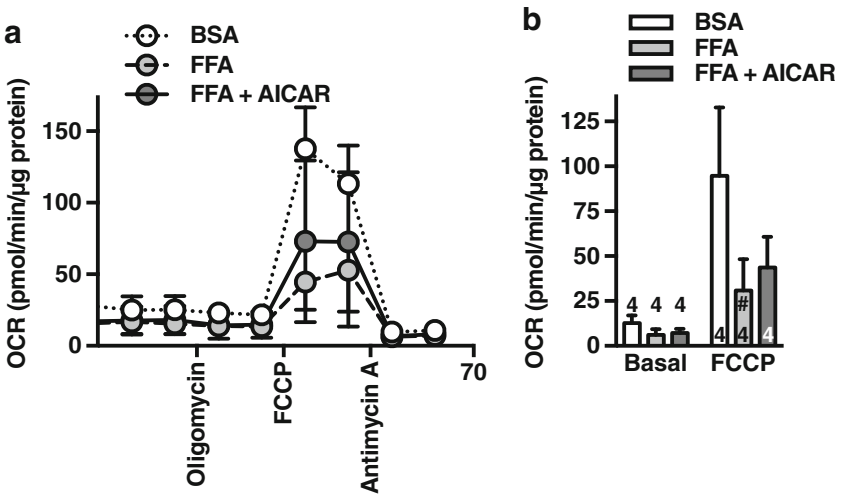


Fig. 6 AICAR does not alter mitochondrial activity in cardiomyocytes exposed to FFA. Cardiomyocytes were cultured for 7 days in medium containing BSA or 0.4 mM free fatty acids (FFA) ± 0.2 mM AICAR. Oxygen consumption rates (OCR) were measured in an extracellular flux analyzer after sequential addition of 10^{-6} M oligomycin, 2×10^{-6} M FCCP, and 10^{-5} M antimycin A to unbuffered DMEM medium containing 1 mM pyruvate and 5 mM glucose. **a** Time-course of one representative experiment; 70 indicates the total duration of the experiment, in minutes. Results are mean ± SD of six to seven wells. **b** Basal and FCCP-induced mitochondrial OCR. Results are mean ± SD of four separate experiments. # : $q < 0.05$ for the effect of FFA

maximal mitochondrial OCR. Thus, the increased glucose uptake and glycolysis in cardiomyocytes exposed to FFA + AICAR cannot be explained by a further impairment of mitochondrial activity.

Effects of chronic AICAR treatment in FFA-exposed cardiomyocytes on PKC expression and signaling

Up to this point, there are strong similarities between the effects of AICAR and tetradecanoyl phorbol acetate (TPA) [52] to improve glucose metabolism and redirect fatty acids to neutral storage in cardiomyocytes exposed to FFA. This prompted us to investigate whether such similarities would extend to protein kinase C signaling, which we showed was markedly altered by chronic TPA treatment. Similar to chronic TPA treatment but to a lesser extent, chronic AICAR treatment, either in absence ($p = 0.0069$ by two-way ANOVA) or in presence of FFA ($p = 0.0004$), resulted in a reduction of phosphorylation of PKC isoforms on residues homologous to T^{410} in PKCζ (Fig. 7a). This result indicated a global decrease in the availability of mature PKC across multiple isoforms (α, β, γ, δ, ε, η, θ, ι). In our previous study, we observed that PKCα, δ, and ε were strongly downregulated upon TPA treatment and that PKCδ was recruited to membrane compartments by chronic FFA exposure, indicating a role for PKCδ activation in the impairment of glucose uptake [52]. In contrast, we found no alterations of PKCα, δ, or ε expression in response to AICAR (Fig. 7b), and PKCδ translocation to the membrane fraction was not prevented by AICAR (Fig. 7c).

Effects of chronic AICAR treatment in FFA-exposed cardiomyocytes on the cytoskeleton

Both microtubules [31] and actin filaments [46] have been shown to be important for glucose transport; we previously observed that cytoskeletal organization was disrupted in cardiomyocytes exposed to FFA [3, 52] but restored in response to the TPA treatment resulting in restoration of glucose uptake [52]. We therefore investigated whether concomitant treatment with AICAR could also improve the cytoskeletal organization in cardiomyocytes exposed to FFA (Fig. 8). As previously described [3], exposure to FFA for 7 days resulted in disorganization of the microtubular and sarcomeric systems, evidenced by immunofluorescent labeling of α-tubulin and α-actinin and F-actin, respectively. Concomitant AICAR treatment did not significantly ameliorate either component of cytoskeletal organization. In fact, treatment with AICAR alone resulted in disorganization of the cytoskeleton.

Discussion

Regulation of glucose metabolism

The first salient finding of this study is the protection to a large extent of insulin- and oligomycin-stimulated glucose transport and glycolysis in cardiomyocytes treated with AICAR in addition to exposure to FFA, as compared to the marked reduction in basal and stimulated glucose transport in cardiomyocytes exposed to FFA alone [3]. We used a supraphysiological concentration of insulin (10^{-6} M) in these experiments to focus our investigations on maximal insulin

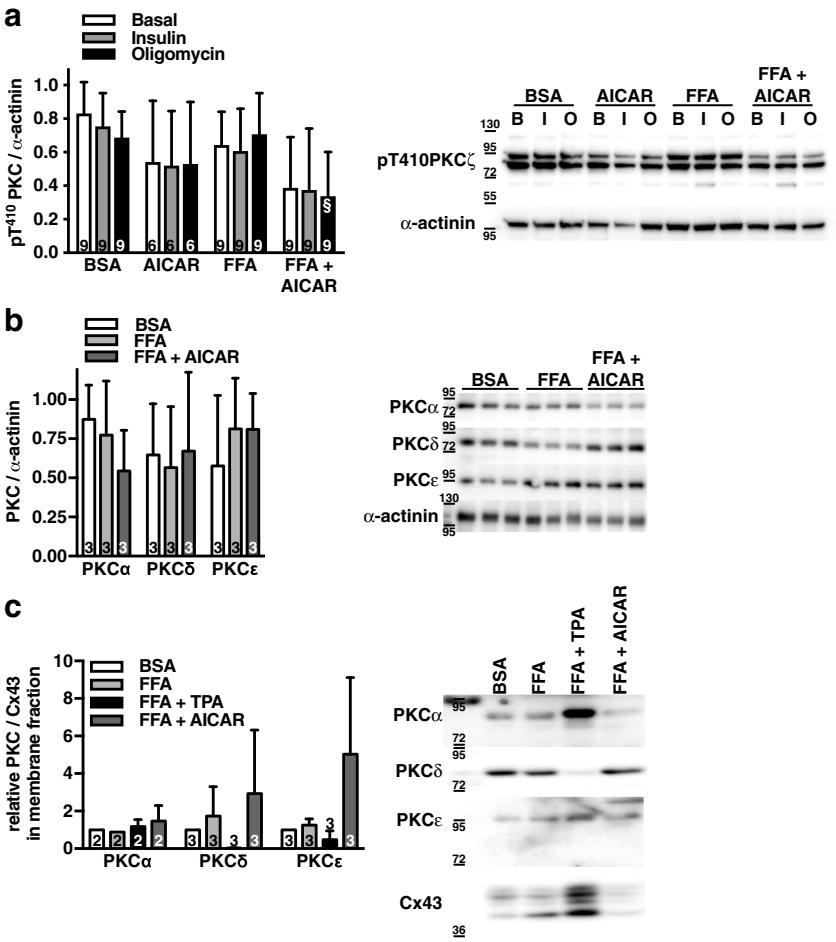


Fig. 7 AICAR reduces activation of some PKC but not translocation of PKCα, δ, or ε. Cardiomyocytes were cultured for 7 days in medium containing BSA or 0.4 mM free fatty acids (FFA) ± 0.2 mM AICAR. **a** Cardiomyocytes were then stimulated for 10 min with 10^{-6} M insulin or for 20 min with 10^{-6} M oligomycin and phosphorylation of PKCs on the site analogous to T^{410} on PKCζ (indicative of activation or preactivation) assessed by Western blot. α-Actinin served as a loading control. **b** Expression of PKCα, PKCδ, and PKCε in whole cell extracts was assessed by Western blot, with α-actinin serving as a loading control. **c** Cardiomyocytes were separated into a cytosol and a membrane fractions and the density of PKC isoforms in the membranes fractions evaluated, with connexin 43 (Cx43) as a loading control for membrane fraction proteins. Results are mean ± SD of the number of separate experiments indicated at the foot of each column. : $q < 0.05$ for the effect of FFA. § : $q < 0.05$ for the effect of AICAR. Right panels: representative Western blots

responsiveness, not insulin sensitiveness, as we previously observed that FFA exposure reduced the former, not the latter, parameter [3].

Inhibition of glucose uptake by an AMPK agonist, as observed in cardiomyocytes under treatment with AICAR alone, may seem paradoxical; it has however been previously reported, albeit not in a chronic exposure setting. Ségalen et al. reported that stimulation of cardiac myocytes with 2 mM AICAR for 40 min reduced basal and insulin-stimulated glucose uptake [44]; this effect was found to be AMPK-independent and explained by intracellular acidification. Also, the chronic effects of AICAR to reduce insulin-stimulated glucose transport in control cardiomyocytes but improve oligomycin-stimulated glucose transport in FFA-exposed cardiomyocytes are akin to the chronic effects of PPARδ stimulation on glucose transport in cardiomyocytes [3], which would concur with the finding that AICAR could stimulate PPARδ-dependent transcription [32]. However, inhibition of PPARδ with GSK3787 did not alter glucose uptake in cardiomyocytes exposed to FFA + AICAR (data not shown). Finally, the cytoskeletal disorganization brought about by chronic treatment with AICAR alone (Fig. 8) may have contributed to the reduction of glucose uptake.

One important point made by Fig. 1a is that improvement of glucose transport by AICAR in the presence of FFA is not related to a general stimulation of glucose transport regardless of the presence or absence of FFA. It is therefore pertaining to mechanisms existing only in the presence of FFA.

The slightly improved expression of the glucose transporters GLUT1 and GLUT4 could certainly contribute to the amelioration of glucose uptake and glycolysis in cardiomyocytes exposed to FFA + AICAR. One should however not overestimate the importance of GLUT expression in determining glucose uptake, as Kaczmarczyk et al. have

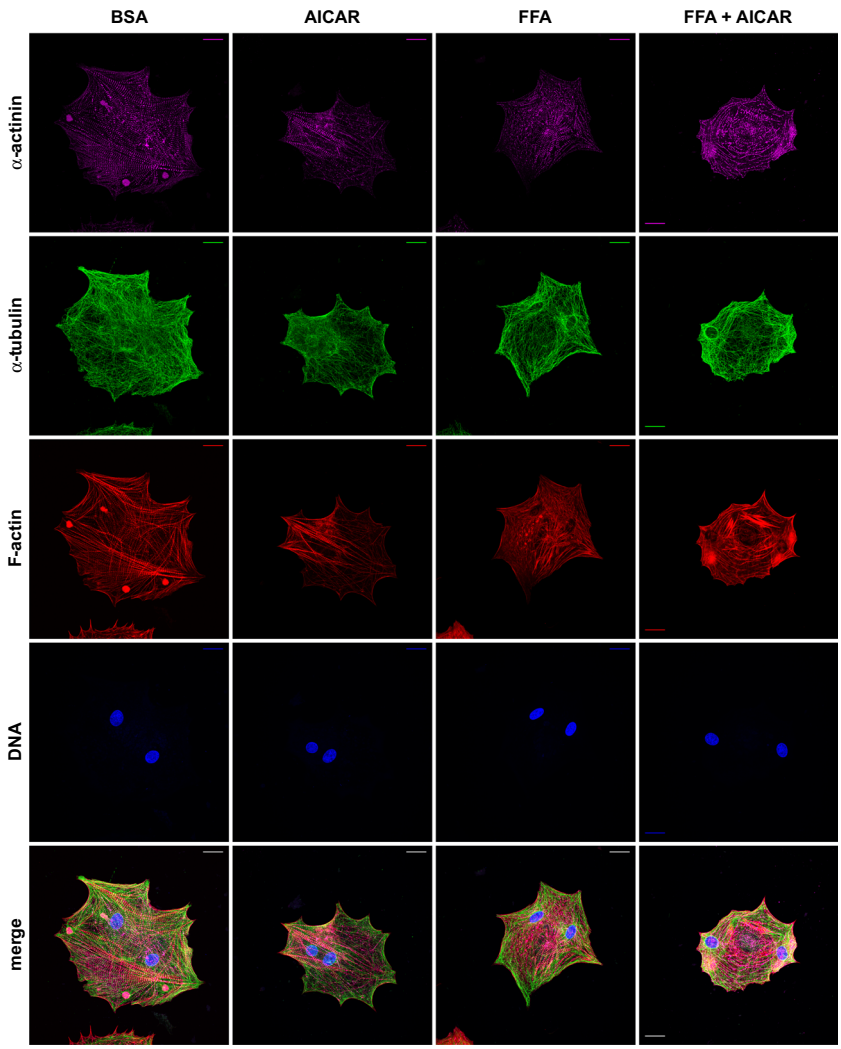


Fig. 8 AICAR does not improve cytoskeleton disorganization. Cardiomyocytes were cultured for 7 days in medium containing BSA or 0.4 mM free fatty acids (FFA) ± 0.2 mM AICAR. Cardiomyocytes were then fixed, permeabilized, and stained with antibodies against sarcomeric α-actinin (magenta) and α-tubulin (green). F-actin was labeled with phalloidin (red). DNA counterstaining (blue) was obtained with DAPI. Scale bar 20 μm. Images are representative of ten microscopic frames over two experiments for each condition

shown that above a quite low threshold of expression, glucose uptake in murine myocardium was independent of GLUT4 expression [23].

AMPK signaling

Examining AMPK signaling in relationship with glucose transport in cardiomyocytes exposed to AICAR, FFA, or both together, we made the puzzling observations that phosphorylation of AMPKα on T^{172}, a classical marker of AMPK activation, was very low, to the point of being almost undetectable in some experiments. This unexpected effect was observed with two distinct antibodies (listed in Table 1), thus is unlikely to be an artifact. The antibodies used to detect phosphoT^{172}-AMPKα and total AMPKα were capable of detecting both α1 and α2 isoforms; therefore, the disappearance of phosphoT^{172}-AMPKα was unlikely to be due to a switch in isoform expression. It remains possible that the expression of β or γ subunits of AMPK was massively reduced under AICAR treatment, thereby preventing the assembly of functional AMPK complexes. This appears to be unlikely, as expression of all three subunits is required for the stability of the AMPK complex; therefore, massively reduced expression of β or γ subunits would induce degradation of the normally expressed α subunit [11]. If this were nevertheless the case, it would mean that the stimulation of glucose uptake by oligomycin observed in cardiomyocytes exposed to FFA + AICAR was not due to AMPK activation but to some other mechanism. Luiken et al. reported that oligomycin activated protein kinase D (PKD, also known as PKCμ) in an AMPK-independent manner and that activated PKD participated in the stimulation of glucose uptake by oligomycin in cardiomyocytes [27]. It is thus possible that the remaining stimulation of glucose uptake by oligomycin in condition with chronic AICAR exposure and apparent deactivation of AMPK was entirely due to PKD activation. On the other hand, chronic AICAR treatment might obscure or prevent AMPKα phosphorylation while maintaining AMPK activity by allosteric activation induced by accumulation of the AICAR metabolite ZMP. However, a recent study of AMPK activation

concluded that T^{172} phosphorylation was required for AMPK activity in a cellular context, even though it could be dispensable for allosteric activation in cell-free extracts [54].

In order to differently assess the activity of AMPK, we determined the phosphorylation status of two of its known substrates, acetyl-CoA carboxylase (ACC) [34] and raptor [17]. Phosphorylation of ACC on S^{79} proved to be a rather insensitive readout of AMPK activation in this model; however, the statistically significant increased overall phosphorylation of S^{79}-ACC in response to AICAR, either in the presence or absence of FFA, suggested that AMPK was being activated by chronic AICAR exposure despite the disappearance of T^{172}-AMPKα phosphorylation.

On the other hand, phosphorylation of raptor on S^{792} displayed a pattern that was consistent with a reduction of AMPK activity in cardiomyocytes exposed to FFA and an improvement of AMPK activity in cardiomyocytes exposed to FFA and AICAR together. These data again argue for AMPK being activated by chronic AICAR exposure despite the disappearance of T^{172}-AMPKα phosphorylation. Furthermore, phosphoS^{792}-raptor data suggest that AMPK activity is low in cardiomyocytes exposed to FFA alone, despite levels of T^{172}-AMPKα phosphorylation similar to those in control cardiomyocytes. Inhibition of AMPK activity has indeed been observed in neonatal rat cardiac myocytes [20] or mouse myotubes [43] following long (16–20 h) incubation with the fatty acid palmitate but, unlike in our study, was associated with reduced T^{172}-AMPKα phosphorylation and prevented by coincubation with oleate.

The pattern of S^{792}-raptor phosphorylation across the different cardiomyocyte treatments matched that of glucose uptake much better than that of T^{172}-AMPKα did. These data should however not be interpreted as indicating that inhibition of mTORC1 via raptor phosphorylation stimulates glucose uptake; indeed, Ginion et al. have shown that mTORC1 inhibition had no effect on glucose transport in adult cardiomyocytes [15]. If anything, inhibition of mTORC1 activity has rather been associated with a reduction of glucose transport in myoblasts [25] or in adipocytes [35].

Whether AICAR actually induces the phosphorylation of T^{172}-AMPKα and activates AMPK in the myocardium is controversial. While Marsin et al. found no activation of AMPK in the rat heart perfused with 1 mM AICAR [29], Russell et al. observed increased AMPK activity and glucose uptake in rat ventricular papillary muscles incubated with 1 mM AICAR [41]. The reason for these discrepancies is not clear. We observed only a slight increase in T^{172}-AMPKα phosphorylation and no increase in phosphoS^{792}-raptor after stimulation with AICAR for 1 h. Thus, it appears that, at least during short exposure to 0.2 mM AICAR, isolated cardiomyocytes do not take up enough of the compound to sufficiently increase intracellular ZMP concentrations for significant AMPK activation. It remains however possible that longer, chronic administration of AICAR leads to sufficient ZMP accumulation for significant AMPK activation to occur.

To determine whether stimulation of glucose uptake by oligomycin under chronic FFA + AICAR was actually AMPK-mediated, we measured glucose uptake and raptor phosphorylation in the presence of the AMPK inhibitor compound C. Though the specificity of CC is not perfect, these findings strongly suggest that AMPK is actually activated by oligomycin treatment in the FFA + AICAR condition, despite phosphoT^{172}-AMPKα being barely detectable.

Lipid metabolism

These results on lipid metabolism in cardiomyocytes exposed to FFA + AICAR are at odds with the expected effects of AMPK activation, which were that fatty acid oxidation would be increased rather than decreased [6]. On the other hand, these authors exposed cardiomyocytes to AICAR for only 2 h, whereas other investigators observed that a longer exposure of hepatocytes to AICAR (24 h) resulted in a decrease in PPARα transcriptional activity that could explain the reduced fatty acid oxidation [45]. Further to above speculative discussion that some of the effects of chronic AICAR could be due to PPARδ activation, we previously observed that selective PPARδ activation also induced lipid droplets biogenesis to a large extent (unpublished observations).

PKC signaling

We previously observed that chronic exposure to the phorbol ester TPA together with FFA improved FFA-impaired glucose uptake and glycolysis in cardiomyocytes [52], similar to our current findings with AICAR. The similarities between the effects of TPA and AICAR extend to lipid metabolism, with reduced fractional oxidation of fatty acids and increased biogenesis of lipid droplets. Also, both TPA and AICAR reduced the general expression of active or activatable PKC. However, whereas TPA reduced expression of PKCα, δ, and ε and prevented activation of PKCδ, AICAR exerted none of these specific effects. There are three possibilities for the interpretation of these results: (1) the reduction in phosphoT^{410}-PKCζ expression is relevant for restoration of glucose uptake in response to both AICAR and TPA, whereas the translocation of PKCδ is an epiphenomenon. In this case, the identity of the culprit PKC isoform would remain unknown. However, amelioration of glucose uptake with a PKCδ inhibitor [52] speaks against this interpretation. (2) The reduction in phosphoT^{410}-PKCζ is an epiphenomenon in both situations, linked to the changes in the metabolic pattern of fatty acids, and TPA and AICAR act via different mechanisms. Indeed, funneling of fatty acids into neutral storage could reduce the intracellular concentration of diacylglycerols, physiological PKC activators. However, inhibition of lipid droplets biogenesis with

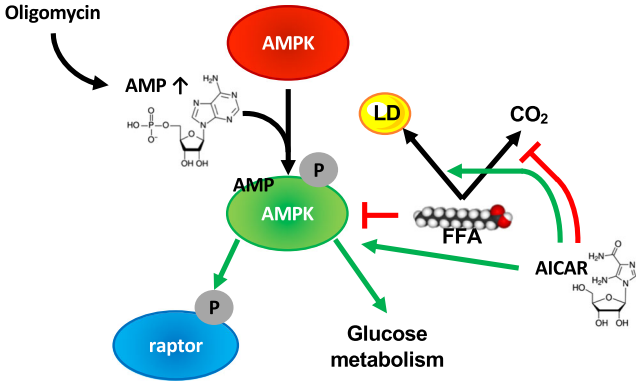


Fig. 9 Summary of main findings and interpretation. In control cardiomyocytes, metabolic stress, as induced by oligomycin and resulting in AMP accumulation, activates AMPK, which stimulates glucose metabolism and phosphorylates raptor. Chronic exposure to free fatty acids (FFA) markedly reduces both AMPK-mediated effects. Chronic AICAR treatment in addition to FFA channels FFA to lipid droplets (LD), reduces FFA oxidation, and restores AMPK-mediated effects

diacylglycerol acyl transferase 1 inhibitors did not prevent the reduction in phosphoT^{410}-PKCζ (preliminary data, not shown). (3) Both the reduction in phosphoT^{410}-PKCζ expression and the translocation of PKCδ are relevant for the restoration of glucose uptake; the fact that only the first one occurs in response to AICAR then potentially explains why the restoration of glucose uptake is only partial with AICAR while it is complete with TPA. Speculatively, this would reinforce the idea put forward earlier that the effects of chronic low-dose AICAR might not be solely related to the activity of AMPK.

Cytoskeletal architecture

Integrity and plasticity of the cytoskeleton play a crucial role for the stimulation of glucose transport in cardiomyocytes [31, 46]. In this study, we confirmed the cytoskeletal disarray previously observed in cardiomyocytes exposed to FFA; AICAR co-treatment did not improve the cytoskeletal architecture. This perhaps also partly explains why amelioration of glucose uptake with AICAR in FFA-exposed cardiomyocytes is only partial. It also suggests that the improvement in cytoskeletal organization observed in response to TPA is independent of the effects shared by TPA and AICAR treatments, i.e., fatty acid metabolism and phosphoT^{410}-PKCζ reduction. Dyntar et al. observed myofibrillar disarray in cardiomyocytes exposed to palmitate and linked it to the biosynthesis of ceramides [12]. Promotion of triglyceride synthesis, as occurs under treatment with FFA + AICAR, reduces ceramide biosynthesis [26] and should therefore lead to the preservation of cytoskeletal organization. However, this may have been prevented by the cytoskeletal disorganization effect of AICAR by itself.

About the experimental model

Adult rat cardiomyocytes in long-term primary culture under high FCS conditions, the model used in this study, undergo dedifferentiation and redifferentiation, resulting after 7–8 days in a phenotype with high myofibrillar organization, contractility [13], and a glucose transport response to insulin and metabolic stress almost identical to that observed in freshly isolated cardiomyocytes [31, 39], despite extensive cytoskeletal reorganization leading to the loss of the rod-like shape. In addition, inclusion of 9-*cis* retinoic acid to the culture medium markedly reduces the extent of transient dedifferentiation and of subsequent hypertrophy [30, 31]. We therefore believe this experimental model to be valid for investigations on the chronic impact of alterations of the extracellular environment on glucose metabolism in cardiomyocytes.

Limitations

Obviously, the main limitation of the present study is that it remains difficult to conclude whether amelioration of glucose metabolism by chronic AICAR co-treatment with FFA exposure is or is not AMPK-mediated. Chronic pharmaceutical inhibition of AMPK activity or AMPK knock-down during AICAR exposure could have resolved this issue, but unfortunately, neither approach proved feasible in our model.

Conclusions

In conclusion (Fig. 9), chronic AICAR treatment of cardiomyocytes restores a normal pattern of substrate metabolism in a context of excessive lipid supply, by redirecting fatty acids to neutral storage. A similar maneuver in vivo in type II diabetes could improve the impaired metabolic stress stimulation of myocardial glucose transport and thus render the diabetic heart less susceptible to ischemic injury.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

The ethical committee of the Geneva University School of Medicine and the Geneva State Veterinary Office approved the study protocol (authorization no. GE/51/16 and no. GE/217/17), which conforms to the

Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

This article does not contain any studies with human participants performed by any of the authors.

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