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RESEARCH ARTICLE

Energetics and Metabolism

Enhanced protein acetylation initiates fatty acid-mediated inhibition of cardiac glucose transport

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Abstract

Fatty acids (FAs) rapidly and efficiently reduce cardiac glucose uptake in the Randle cycle or glucose-FA cycle. This fine-tuned physiological regulation is critical to allow optimal substrate allocation during fasted and fed states. However, the mechanisms involved in the direct FA-mediated control of glucose transport have not been totally elucidated yet. We previously reported that leucine and ketone bodies, other cardiac substrates, impair glucose uptake by increasing global protein acetylation from acetyl-CoA. As FAs generate acetyl-CoA as well, we postulated that protein acetylation is enhanced by FAs and participates in their inhibitory action on cardiac glucose uptake. Here, we demonstrated that both palmitate and oleate promoted a rapid increase in protein acetylation in primary cultured adult rat cardiomyocytes, which correlated with an inhibition of insulin-stimulated glucose uptake. This glucose absorption deficit was caused by an impairment in the translocation of vesicles containing the glucose transporter GLUT4 to the plasma membrane, although insulin signaling remained unaffected. Interestingly, pharmacological inhibition of lysine acetyltransferases (KATs) prevented this increase in protein acetylation and glucose uptake inhibition induced by FAs. Similarly, FA-mediated inhibition of insulin-stimulated glucose uptake could be prevented by KAT inhibitors in perfused hearts. To summarize, enhanced protein acetylation can be considered as an early event in the FA-induced inhibition of glucose transport in the heart, explaining part of the Randle cycle.

NEW & NOTEWORTHY Our results show that cardiac metabolic overload by oleate or palmitate leads to increased protein acetylation inhibiting GLUT4 translocation to the plasma membrane and glucose uptake. This observation suggests an additional regulation mechanism in the physiological glucose-FA cycle originally discovered by Randle.

acetylation; fatty acids; glucose uptake; insulin; Randle cycle

INTRODUCTION

Heart is a metabolic omnivore organ able to catabolize a variety of substrates to generate its energy (1–3). The use of those different substrates is tightly regulated to allow an optimal contractile function. Alteration of cardiac metabolic flexibility is associated with several cardiovascular diseases, such as heart failure and diabetic cardiomyopathy (DCM) (4–7). A better comprehension of the regulatory mechanisms ruling cardiac metabolic flexibility is therefore crucial to prevent the development of such pathologies. The two main energy-providing metabolites used by the heart are fatty acids (FAs, 60%–80%) and carbohydrates (40%–20%) (8). Randle and colleagues (9) demonstrated in 1963 that FA and glucose metabolisms are mutually exclusive, allowing heart metabolism to constantly adapt to the fluctuations of these substrates throughout the day, a regulation known as the Randle cycle. The inhibition of glucose metabolism by FA catabolism has been attributed to three main regulatory points: glucose oxidation, glycolysis, and glucose transport (10). The inhibition of pyruvate dehydrogenase (PDH, controlling glucose oxidation) and phosphofructokinase 1 (regulating glycolysis) have been well documented. However, the suppression of glucose transport by FAs remains debated and poorly understood. Indeed, Randle initially suggested that PDH was the main inhibitory site of FAs and that accumulation of glycolytic intermediates could explain glucose uptake inhibition (9). More recent studies argued that PDH inhibition only accounts for a minor part

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of glucose transport inhibition and that the initial event occurs at the level of glucose transport itself (11, 12).

Glucose uptake by cardiomyocytes mainly depends on two transporters, glucose transporters 1 and 4 (GLUT1 and GLUT4) (13, 14). GLUT1 is constitutively located at the plasma membrane and is partially responsible for basal glucose uptake. On the other hand, GLUT4 is stored in cytoplasmic vesicles named GLUT4 storage vesicles (GSVs) (15, 16). When glycemia rises in a postprandial state, pancreatic release of insulin promotes the activation of the protein kinase B (PKB)/Akt pathway inducing the translocation of GSVs to the periphery of cardiomyocytes where they fuse with the plasma membrane (13, 17). This stimulation causes a significant increase in glucose absorption as well as a dramatic metabolic rearrangement toward glucose consumption. It has long been understood that the quick loss of glucose transport stimulation in the presence of high FA concentrations leads to the heart's preferential usage of FAs (18). Despite insulin resistance being a major feature of type 2 diabetes leading to chronic inhibition of glucose metabolism, it remains unclear what mechanisms are involved in the acute inhibition of glucose metabolism. In line with this, recent research suggests that protein acetylation on lysine may play a role in the early reduction of insulin-stimulated glucose absorption (19–21).

Lysine acetylation is a posttranslational modification that involves the addition of an acetyl group from acetyl-CoA to the side chain of particular protein lysine residues (22, 23). Although protein acetylation can occur spontaneously, this reaction is largely mediated by lysine acetyltransferases (KATs) while lysine deacetylation is catalyzed by deacetylases. Deacetylases are divided into two subgroups, the KDACs (for lysine deacetylases, previously named histone deacetylases, HDACs) that use Zn^{2+} as a cofactor and the sirtuins (SIRTs), which are NAD^{+}-dependent deacetylases. Several studies highlighted a link between protein acetylation and cardiac metabolism, especially in the context of type 2 diabetes characterized by increased FA metabolism and repressed glucose metabolism (19, 21, 24–26). Previously, our group discovered that leucine and ketone bodies, two cardiac metabolic substrates elevated in diabetes, inhibit GLUT4 translocation by causing a global rise in acetyl-CoA production that leads to increased protein acetylation (20). In the present work, we show that oleate and palmitate metabolism, which also produces acetyl-CoA, induces a rapid rise in protein acetylation that inhibits the insulin-mediated translocation of GLUT4. This finding suggests that increased protein acetylation brought on by FAs may play a significant role in the Randle cycle and could contribute to the heart's exclusive usage of FAs throughout the evolution of type 2 diabetes.

MATERIAL AND METHODS

Animal care

All animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and approved by local authorities (Comité d'éthique Facultaire pour l'Expérimentation Animale, 2016/UCL/MD/027 and 2021/UCL/MD/015).

FAs-Bovine Serum Albumin Complex Preparation (5:1)

For effective FA distribution, FAs must be complexed with bovine serum albumin (BSA). To prepare a 20 mL solution of 10 mM complexed FA, 56.5 mg of oleic acid (Merck, Cat. No. O1008) or 51.3 mg of palmitic acid (Merck, Cat. No. P0500) was dissolved in 10 mL of warm (70°C) 0.9% (wt/vol) NaCl solution. Simultaneously, 2.45 g FA-free BSA (FAF-BSA) (Merck, Cat. No. 126575) was dissolved in 7 mL of warm (37°C) 0.9% (wt/vol) NaCl. Then, FA solution was slowly added to stirring BSA solution (37°C) to form FA-BSA complexes before adjusting the pH solution to 7.4 with 1 M NaOH. The volume of the solution was finally adjusted to 20 mL with 0.9% (wt/vol) NaCl before aliquoting and storing at −20°C. The final concentrations of FAs used in this study were obtained from a dilution of this stock solution in medium 199 (Gibco, Cat. No. 31150022).

Primary Culture of Adult Rat Cardiomyocytes

Isolation of rat cardiomyocytes was performed as previously described (27). Hearts from 250-g Wistar male rats (in-house breeding) were excised, canulated by the aorta, and retrogradely perfused with Ca^{2+}-free Krebs-Henseleit buffer, consisting of (in mM) 5 glucose, 2 pyruvate, 10 HEPES (pH 7.4), and 150 NaCl, to remove residual blood. Hearts were then digested by adding 0.2 mM Ca^{2+}, 1 mg/mL type 2 collagenase (Worthington, Cat. No. LS004177), and 0.4% (wt/vol) FAF-BSA. Once digested, hearts were mechanically disrupted with scissors. The suspension was then incubated for 10 min under continuous stirring before a progressive reintroduction of Ca^{2+} to reach a final concentration of 1 mM. Finally, cardiomyocytes were isolated by sedimentation before their plating in medium 199 (Gibco, Cat. No. 31150022) on laminin (Merck, Cat. No. L2020)-coated 3-cm dishes or laminin-coated glass coverslips for immunostaining experiments. Medium was then replaced 1 h later with medium-199 supplemented with 2 mM carnitine, 5 mM creatine, 5 mM taurine, 10^{-10} M T3, 0.2% (wt/vol) FAF-BSA, and antibiotics. After 1-h incubation at 37°C and 5% CO_2, cardiomyocytes were used for experiments.

Cardiomyocyte Treatment

Rat cardiomyocytes were treated for 20 h with FA-BSA complexes at the concentrations specified in the figure legends or with an equivalent BSA concentration (vehicle) with or without a 15-min preincubation with 25 μM anacardic acid (Merck, Cat. No. A7236), an inhibitor of lysine acetyltransferases. Insulin (Novo Nordisk, Actrapid 100 IU/mL) was added in the medium for the last 30 min at a concentration of 3×10^{-9} M, while 1 μM of the AMP-activated protein kinase (AMPK) activator oligomycin (Merck, Cat. No. 75351) was added 1 h before the end of the experiment. To evaluate glucose uptake, cells were incubated for the last 30 min with 0.2 μCi/mL [2-^{3}H]-glucose (Perkin Elmer, Cat. No. NET238C001MC) after which 1 mL of culture medium was collected for further analysis. Cells were then lysed in 90 μL of cold lysis buffer, consisting of 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 1% (vol/vol) Triton X-100, 20 mM glycerol-2-phosphate, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.5 mM PMSF, 1 mM benzamidine-HCl, 1 μg/mL leupeptine, 1 μg/mL pepstatin A, 1 mM DTT, 1 mM Na_3VO_4,

10 μM EX527, and 1 μM TSA, for immunoblotting after protein quantification by the Bradford method.

Rat-Heart Langendorff Perfusion Model

After anesthesia, rat hearts were excised from 250-g Wistar male rats (in-house breeding), cannulated via the aorta on a Langendorff apparatus, and perfused 10 min with Krebs-Henseleit buffer composed of (in mM) 110 NaCl, 4.7 KCl, 1.25 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5.5 glucose, and 1.5 $CaCl_2$, continuously gazed with 95% O_2-5% CO_2 for equilibration. Hearts were preincubated for 5 min with or without 10 μM garcinol (ChemCruz, Cat. No. sc-200891), a general acetyltransferase inhibitor, and then treated for 30 min with the same buffer supplemented with 800 μM of oleate, palmitate, or an equivalent quantity of FAF-BSA as vehicle. After 10 min of treatment, [2-^{3}H]-glucose was added to the medium with or without 3×10^{-9} M insulin. Eluates (1 mL) were collected every 5 min during 20 min to assess glucose uptake by the hearts.

Glucose Uptake Measurement

Glucose uptake was measured as previously described (27). Samples (1 mL of cellular culture medium or of perfused heart eluate) were deproteinized on ice by 6.4% (vol/vol) perchloric acid (PCA) precipitation and centrifuged at 4°C, 422 *g* for 10 min. Supernatant (900 μL) was then neutralized with a 3 M $KOH/KHCO_3$ solution. The volume was then adjusted to 1.5 mL with water before centrifugation at 4°C, 422 *g* for 10 min. After that, 1 mL of supernatant was collected and added to 1 mL water before passing through a gravity flow chromatography column of borate-coupled AG 1-X8 resin (Biorad, Cat. No. 140–1453) to eliminate unmetabolized [2-^{3}H]-glucose. The radioactivity in the column flow-through was measured with a scintillation counter and corresponds to ^3H_2O generated by [2-^{3}H]-glucose metabolization which is proportional to [2-^{3}H]-glucose uptake. Glucose uptake in cardiomyocytes was then normalized against total radioactivity added to the medium and protein content of each culture dish. Glucose uptake in perfused hearts was normalized against total radioactivity in the perfusion medium, heart weight, and perfusion output.

Evaluation of GLUT4 Translocation

Primary cultured adult rat cardiomyocytes were plated on glass coverslips and incubated with adenoviral particles designed to induce expression of hemagglutinin (HA)-GLUT4-green fluorescent protein (GFP), a construct kindly provided by Dr. Corley Mastick (Dept. of Biochemistry and Molecular Biology, Univ. of Nevada, Reno, NV). GFP fused to the carboxyl extremity monitors exogenous GLUT4 expression and HA addition in the first extracellular loop of the transporter allows immunodetection of GLUT4 when inserted into the cellular membrane of nonpermeabilized cardiomyocytes. After 48 h, cells were fixed for 10 min at room temperature in 4% (wt/vol) paraformaldehyde (PFA) without any permeabilization. Fixed cardiomyocytes were then rinsed three times with PBS and incubated for 30 min in 5% (wt/vol) BSA-PBS blocking solution. Immunolabeling of HA epitope of membrane GLUT4 was achieved by a 1-h incubation with a primary anti-HA antibody diluted in 1% (wt/vol) BSA-PBS followed by a 1-h incubation with an anti-rabbit antibody coupled to Alexa Fluor 568 diluted in 1% (wt/vol) BSA-PBS. All antibody specifications used for immunofluorescent studies are available in Supplemental Table S1 (see https://doi.org/10.6084/m9.figshare.19948532). Coverslips were then mounted on microscope slides with Vectashield mounting medium (Vectorlabs, Cat. No. H-1000). Red fluorescence coming from Alexa Fluor 568 and green intrinsic fluorescence of GFP were visualized under a Zeiss Imager.Z1 microscope equipped with an ApoTome device. The red fluorescence (HA staining) signal was quantified by ImageJ software relative to the GFP signal to reflect GLUT4 translocation to the plasma membrane.

Immunoblot Analysis

Protein lysates were centrifuged (3,500 *g*, 10 min, 4°C) and, after Bradford protein concentration measurement, soluble protein lysates were used to study protein expression, phosphorylation, and acetylation levels by immunoblotting. Proteins (20 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred on a polyvinylidene difluoride (PVDF) membrane. Membranes were then blocked for 1 h in 5% milk/Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBS-T) and incubated overnight with primary antibody diluted in 5% milk/TBS-T. After three washings of 10 min with TBS-T, membranes were incubated for 1 h at room temperature with the corresponding secondary antibody in 5% milk/TBS-T. All antibody specifications used for immunoblotting analysis are available in Supplemental Table S1. Finally, membranes were washed three times for 10 min in TBS-T and were incubated with enhanced chemiluminescence substrate (Merck, Cat. No. 11500694001). Light emission was captured on X-ray films. Eukaryotic translation elongation factor 2 (eEF2) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was blotted to assess the loading of each gel. Band intensities, reflecting protein abundance, were quantified with ImageJ software and normalized to internal loading control protein (eEF2 or GAPDH) intensity. Analysis of phosphorylated or acetylated proteins involved an additional normalization step toward their own normalized total form. Western blots used to generate figures are presented in their uncropped version in Supplemental Fig. S1 (see https://doi.org/10.6084/m9.figshare.19948610).

Statistics

Results are expressed as means ± SE of at least three independent experiments. Statistical significance was calculated by one- or two-way analysis of variance (ANOVA) with Tuckey's post hoc test. *P* value of 0.05 or less was considered statistically significant. All statistical analyses were performed on GraphPad Prism 9.

RESULTS

Oleate and Palmitate Similarly Inhibit Insulin-Stimulated Glucose Uptake in Adult Cardiomyocytes without Altering Insulin Signaling Pathway

Freshly isolated primary cultured adult rat cardiomyocytes were treated for 20 h with increasing concentrations (0 to 300 μM) of oleate or palmitate, two of the most abundant FAs in plasma that are also described to be elevated

during early diabetes (28–31). To evaluate the effect of FAs on cardiac insulin-stimulated glucose uptake, cells were incubated with 3×10^{-9} M insulin and radiolabeled [2-^{3}H]-glucose during the last 30 min of treatment (Fig. 1*A*). As previously described (13, 20, 32–34), insulin strongly stimulated glucose transport (Fig. 1*B*). Both oleate and palmitate significantly weakened this insulin response at 100 μM and fully abolished insulin-stimulated glucose uptake at 300 μM, two concentrations of FAs that fall within their (patho)physiological plasmatic range (2, 29, 31). We monitored the phosphorylation of PKB/Akt, the key player in the insulin pathway, ribosomal protein S6, which is a downstream target of PKB/Akt signaling, and AS160, a PKB/Akt substrate involved in GLUT4 translocation, to determine whether this effect is the result of a defect in insulin signaling (34). As shown in the immunoblots presented in Fig. 1, *C* and *D*, neither oleate nor palmitate reduced the phosphorylation state of these proteins or their total expression after insulin treatment, at concentrations impairing insulin-stimulated glucose uptake. This highly suggests that the molecular mechanism involved in the FA-induced inhibition of insulin-stimulated glucose uptake occurs downstream AS160, independently of insulin signaling pathway inhibition.

Oleate and Palmitate Inhibit Basal and AMPK-Stimulated Glucose Uptake

To confirm that FAs inhibit glucose uptake independently of an alteration of the insulin signaling, we evaluated the effect of oleate and palmitate on basal glucose transport and glucose transport stimulated by the metabolic sensor AMPK. Similar to the previous experimental procedure, primary cultured adult rat cardiomyocytes were incubated for 20 h with one or the other FA. For the final treatment hour, cells were stimulated or not with 1 μM oligomycin (Fig. 2*A*), which activates AMPK via an increase in cellular AMP-to-ATP ratio by inhibiting complex V of the mitochondrial electron transport chain (ATP synthase). As extensively studied in the past (27, 35), oligomycin efficiently promoted glucose transport (Fig. 2*B*). As for insulin response, we observed that both FAs abolished basal and oligomycin-stimulated glucose uptake (Fig. 2*B*). To rule out any possible alteration of AMPK signaling that could explain decreased glucose uptake upon oligomycin stimulation, we assessed the phosphorylation state of AMPK, acetyl-CoA carboxylase (ACC, a bona fide AMPK substrate), and AS160 (AMPK substrate involved in GLUT4 translocation) by immunoblotting (Fig. 2*C*). Phosphorylation levels in response to oligomycin stimulation and the total expression of these proteins were not decreased by either of the two FAs (Fig. 2*D*). Taken together, these results show that oleate and palmitate inhibit glucose transport irrespective of the stimulus used, suggesting a common inhibitory mechanism independent of insulin and AMPK signaling.

Oleate and Palmitate Impair GLUT4 Translocation to the Plasma Membrane

Glucose transport being mainly driven by GLUT4, we next assessed the effects of oleate and palmitate on its translocation and insertion into the plasma membrane. Freshly isolated adult rat cardiomyocytes were incubated for 48 h (Fig. 3*A*) with adenoviral particles coding for a modified GLUT4 protein in which an HA tag was inserted within an extracellular loop located in the NH_2-terminal region and a green fluorescent protein (GFP) was fused to its COOH-terminal part (HA-GLUT4-GFP, Fig. 3*B*) (36). This double-labeled construct allowed us to monitor *1*) overall exogenous GLUT4 expression thanks to the GFP and *2*) the particular subset of GLUT4 inserted into the plasma membrane through the immunolabeling of nonpermeabilized cells with an anti-HA antibody (20, 24). In agreement with literature (37), insulin-induced relocalization of GLUT4 to the plasma membrane was observed by an increase in HA staining (red) at the periphery of cardiomyocytes (Fig. 3*D*). A preincubation of cardiomyocytes for 20 h with oleate or palmitate (100 and 300 μM) totally prevented GLUT4 translocation upon insulin stimulation (Fig. 3*C*), highly suggesting that FA inhibitory effect occurs at the level of GLUT4 transfer and insertion to the sarcolemmal membrane.

Oleate and Palmitate Induce a Global Rise in Cardiac Protein Acetylation That Mediates Glucose Uptake Inhibition

We have previously reported that an excess of leucine or ketone bodies, elevated in early diabetes, increases protein acetylation in cardiomyocytes and perfused hearts (20). Catabolism of these energy-providing substrates produces a large amount of acetyl-CoA that feeds KATs, promoting global protein acetylation (38). As FAs generate acetyl-CoA through their catabolism, we investigated the impact of oleate and palmitate on protein acetylation levels (Fig. 4*A*). Immunoblotting with a pan anti-acetyl lysine antibody revealed that 300 μM oleate or palmitate induced a significant rise in protein acetylation (Fig. 4, *B* and *C*).

To evaluate the importance of FA-induced increase in protein acetylation in their inhibitory effect on glucose uptake, we cotreated cardiomyocytes with anacardic acid, a broad-spectrum KAT inhibitor (Fig. 4*A*). As shown in Fig. 4, *B* and *C*, preincubation with 25 μM anacardic acid prevented the global increase in protein acetylation induced by oleate or palmitate. In parallel, anacardic acid impeded the inhibitory action of FAs on glucose uptake, maintaining insulin capacity to stimulate glucose uptake despite the presence of oleate or palmitate (Fig. 4*D*). It could be noted that anacardic acid treatment also enhanced basal and insulin-stimulated glucose uptake in the absence of FAs, confirming a link between cardiac protein acetylation and glucose uptake.

KAT inhibitor Counteracts the Inhibitory Effects of Oleate and Palmitate on Insulin-Stimulated GLUT4 Translocation

We next checked if preserved insulin stimulation of glucose transport upon FA treatment by anacardic acid resulted from restored GLUT4 translocation capacity. Cardiomyocytes expressing HA-GLUT4-GFP were treated with 100 and 300 μM of oleate or palmitate in the absence or presence of 25 μM anacardic acid (Fig. 5*A*). Anacardic acid efficiently preserved membrane GLUT4 relocalization upon insulin stimulation despite the presence of oleate or palmitate (Fig. 5, *B*–*D*), confirming the implication of protein acetylation in the inhibition of GLUT4 translocation by FAs.

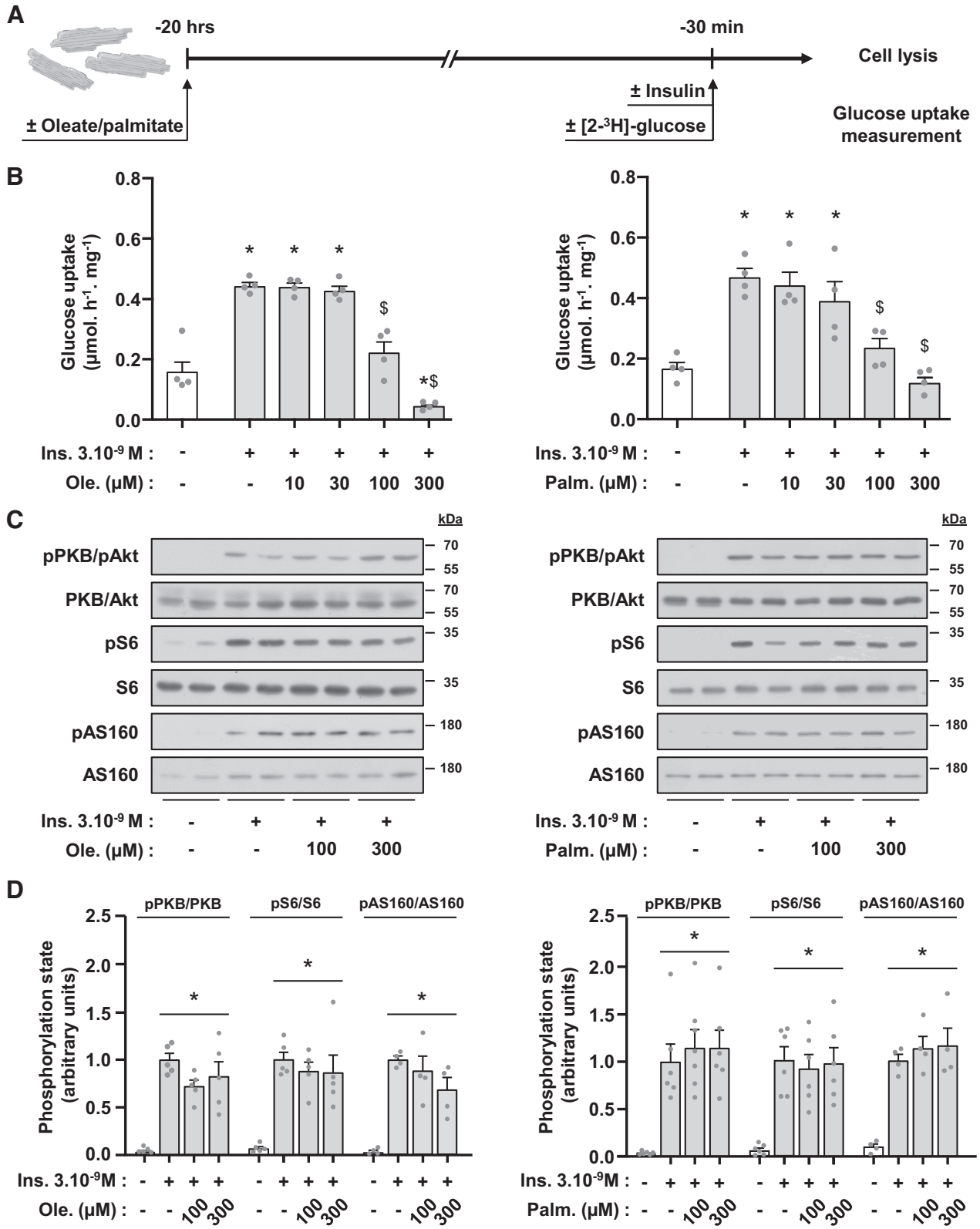


Figure 1. Short-term treatment with oleate (Ole) or palmitate (Palm) inhibits insulin (Ins)-stimulated glucose uptake in adult cardiomyocytes without altering insulin signaling pathway. *A*: experimental design to assess the effect of oleate or palmitate on insulin-stimulated glucose uptake and insulin signaling pathway in primary cultured adult rat cardiomyocytes. *B*: effect of increasing concentrations (0, 10, 30, 100, or 300 µM) of oleate (*left*) or palmitate (*right*) on insulin-stimulated glucose uptake (n = 4 biological replicates per group). Representative immunoblots (*C*) and their quantification (*D*) of the phosphorylated forms of PKB/Akt, S6, and AS160 normalized by their total form obtained from cardiomyocytes treated with oleate (100 or 300 µM, *left*) or palmitate (100 or 300 µM, *right*) (n = 4–6 biological replicates per group). Values are means ± SE. Statistical analysis was conducted by one-way ANOVA. *$P \leq 0.05$, relative to insulin-unstimulated cells; \$$P \leq 0.05$, relative to FA-untreated cells in presence of insulin. FA, fatty acid.

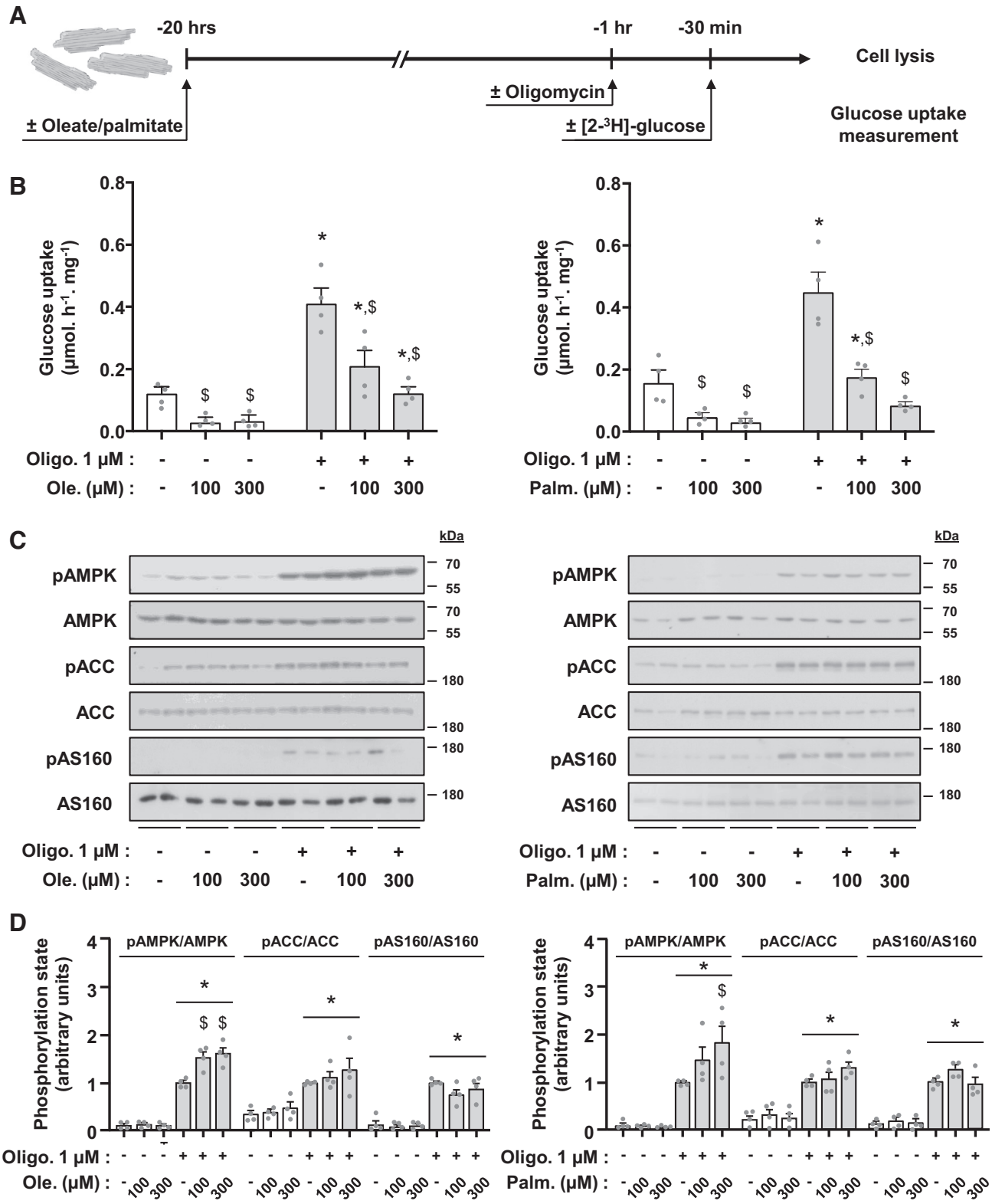


Figure 2. Oleate (Ole) and palmitate (Palm) inhibit basal and AMPK-stimulated glucose uptake. *A*: experimental design to assess the effect of oleate or palmitate on basal glucose uptake, oligomycin (Oligo)-stimulated glucose uptake, and AMPK signaling pathway in primary cultured adult rat cardiomyocytes. *B*: effect of oleate (*left*) or palmitate (*right*) on basal and oligomycin-stimulated glucose uptake. Representative immunoblots (*C*) and their quantification (*D*) of the phosphorylated forms of AMPK, ACC, and AS160 compared with the total forms of the proteins obtained from cardiomyocytes treated with oleate (*left*) or palmitate (*right*). Values are means ± SE of 4 independent experiments. Statistical analysis was conducted by paired two-way ANOVA (*B*) or by unpaired two-way ANOVA (*D*). *$P \leq 0.05$, relative to respective oligomycin-unstimulated cells; \$$P \leq 0.05$, relative to respective FA-untreated cells in presence or in absence of oligomycin. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; FA, fatty acid.

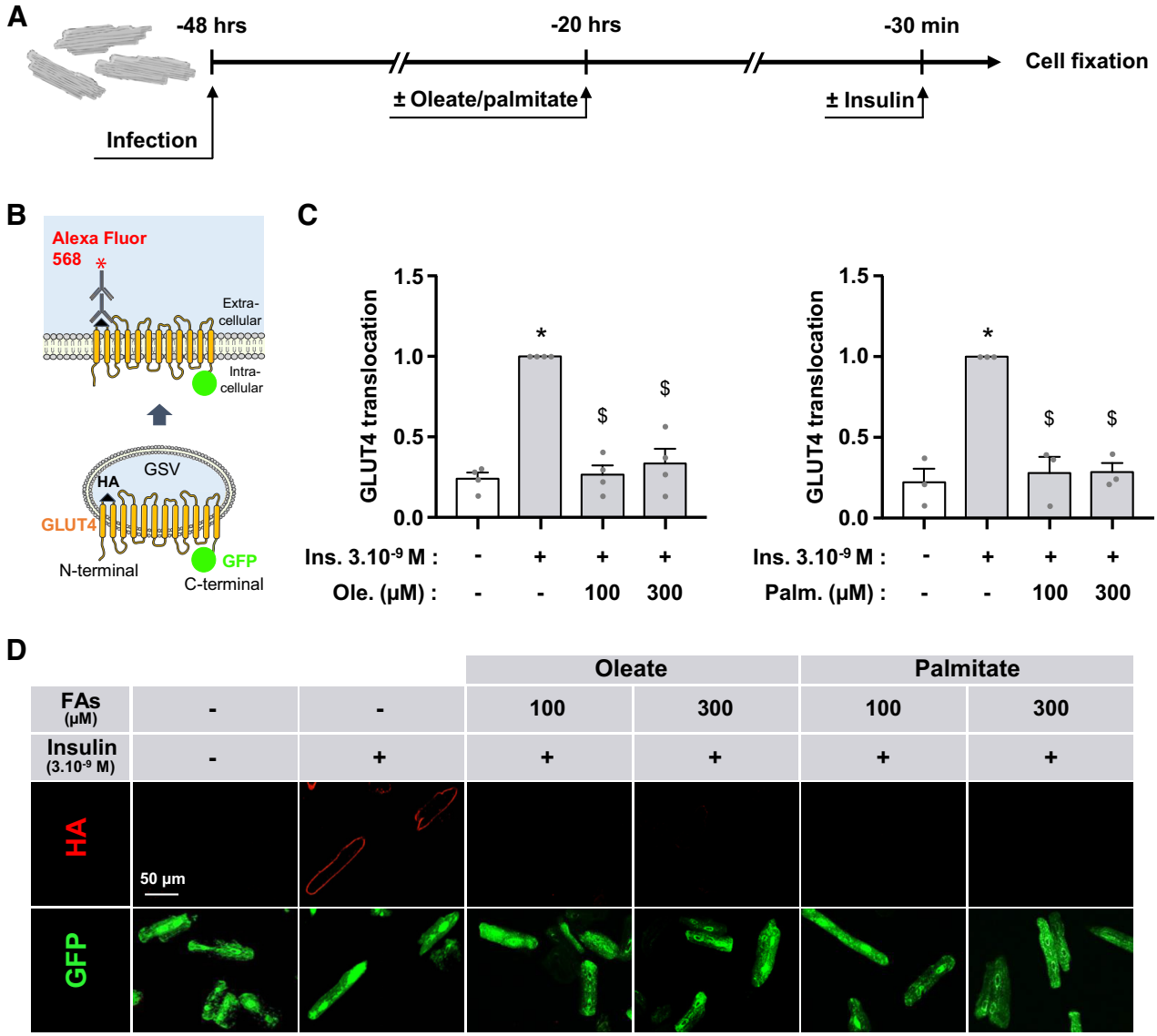


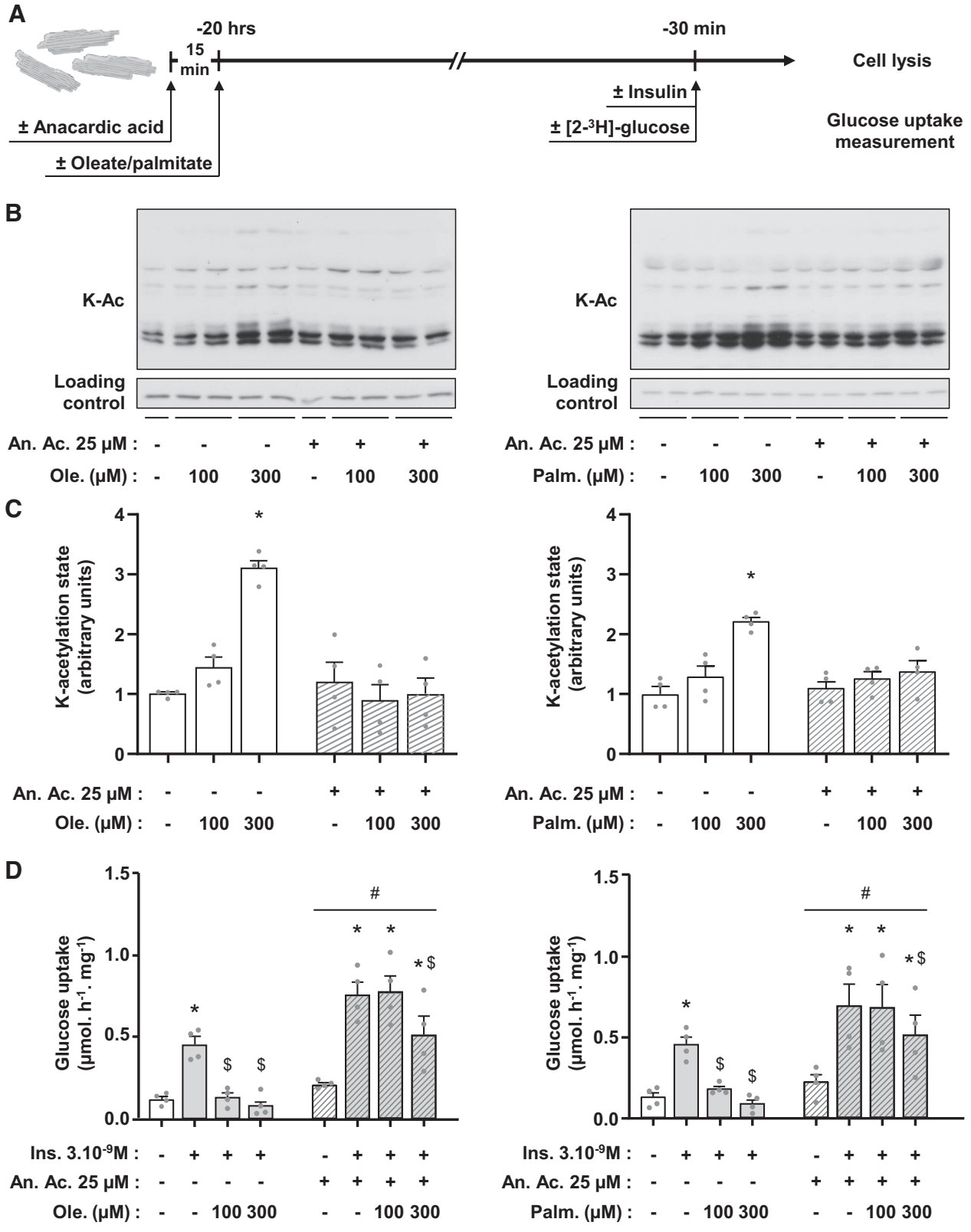
Figure 3. Oleate (Ole) and palmitate (Palm) impair insulin (Ins)-stimulated GLUT4 translocation to the plasma membrane. *A*: experimental design to assess the effect of oleate or palmitate on insulin-stimulated GLUT4 translocation in primary cultured adult rat cardiomyocytes. *B*: schematic representation of exogenous hemagglutinin (HA)-GLUT4-green fluorescent protein (GFP) and antibody accessibility to HA when the chimeric transporter is localized in GLUT4 storage vesicles (GSVs) or at the plasma membrane of nonpermeabilized cardiomyocytes. Quantification (*C*) and representative images of fluorescence microscopy (*D*) of GLUT4 translocation expressed as the ratio of the red channel signal (HA:membrane) on the green channel signal (GFP:total) in nonpermeabilized cardiomyocytes expressing HA-GLUT4-GFP protein and treated with oleate (100 or 300 μM, *left*) or palmitate (100 or 300 μM, *right*). Values are means ± SE of 3 independent experiments and are expressed relative to fatty acid (FA)-untreated cells in presence of insulin. Statistical analysis was conducted by one-way ANOVA. *$P \leq 0.05$, relative to insulin-unstimulated cells; \$$P \leq 0.05$, relative to FA-untreated cells in presence of insulin.

Oleate and Palmitate Inhibition of Insulin-Stimulated Glucose Uptake in Perfused Hearts Is Prevented by KAT Inhibitor

Finally, we wondered if our observations made in freshly isolated cardiomyocytes could be transposed into a more physiological and relevant model such as perfused hearts. For this purpose, we measured glucose uptake in ex vivo beating hearts retrogradely perfused on a Langendorff apparatus. After a 15-min equilibration of the hearts with a Krebs-Ca^{2+} buffer and a 10-min incubation with 800 μM of oleate or palmitate, [2-^{3}H]-glucose was added in the medium to assess glucose uptake in the presence or absence of 3×10^{-9} M insulin (Fig. 6*A*). As previously shown (24), accumulation over the time of ^3H_2O in heart perfusates was significantly higher upon insulin incubation (Fig. 6*B*), reflecting increased glucose uptake (Fig. 6*C*). This stimulation was abrogated by oleate and palmitate. Addition of 10 μM garcinol, another KAT inhibitor, which can be used in perfused heart, was able to prevent the inhibition by oleate or palmitate of insulin-stimulated glucose uptake. This result supports our observations in cultured cardiomyocytes and confirms the implication of protein acetylation in the inhibitory effects of FAs on insulin-stimulated glucose uptake.

DISCUSSION

In the early 60s, Randle and colleagues (9) discovered that FAs are able to inhibit glucose metabolism, an acute regulation known as the "glucose-FA cycle" or "Randle cycle." A few decades of studies have shown that inhibition of glucose use by FAs occurs at several levels including glucose uptake, glycolysis, and its mitochondrial oxidation. Although the molecular mechanisms involved in the regulation of glycolysis and glucose oxidation are well defined, the effects of FAs



on glucose transport are still poorly understood (10). Growing evidence suggests that protein acetylation, which is increased in mouse models of diabetes, might be a crucial factor in cardiac metabolic inflexibility (21, 25, 26, 39). For example, acetylation has been shown to modify mitochondrial glucose and FA oxidation (19). However, as far as we know, protein acetylation has never been suggested to acutely regulate glucose transport downstream FAs. Our group previously demonstrated that leucine and ketone bodies, alternative substrates of the heart, inhibit insulin-stimulated cardiac glucose uptake via a protein acetylation-dependent process, possibly caused by excessive acetyl-CoA production (20). Here, we show that FAs, which also generate a substantial amount of acetyl-CoA, could act similarly, indicating that protein acetylation is an important and newly identified mechanism participating in the Randle cycle.

It is interesting to highlight that both oleate and palmitate exerted analogous effects on cardiac glucose transport despite noticeable differences in terms of structure and reputation. Oleate is a monounsaturated FA of 18-carbon atoms (C18:1 cis-9) with many beneficial effects reported, whereas palmitate is a saturated FA with a 16-carbon backbone (C16:0) described as detrimental fat (40, 41). Like several other saturated FAs, palmitate is implicated in adverse events such as alteration of the insulin signaling pathway, accumulation of lipotoxic intermediates, and increased reactive oxygen species (ROS) production. However, no difference was observed between the two FAs in our experimental setting, suggesting that the deleterious abovementioned effects attributed to saturated FAs are not the cause of the acute inhibition of glucose uptake. The explanation for this analogous action of both FAs might come from the fact that their mitochondrial β-oxidation could similarly lead to a rapid increase in acetyl-CoA pool promoting comparable protein acetylation and participating in the Randle cycle. As a result, all cardiac energy-producing substrates that generate acetyl-CoA could potentially change glucose absorption via protein acetylation, as we have shown for FAs in this study and for ketone bodies and leucine in earlier work (20).

FAs, as well as ketone bodies and leucine, have also been described to be increased in the blood of prediabetic patients (31). One could therefore hypothesize that an elevation of these substrates results in a sustained increase in cardiac protein acetylation and therefore participates in the establishment of the metabolic inflexibility of the diabetic heart via an exacerbated Randle cycle. This is supported by the observation that protein acetylation is also increased in the diabetic heart (19, 21, 24). Therefore, further exploration of protein acetylation involvement in the early events of diabetic cardiomyopathy represents a stimulating future challenge. This could eventually lead to new therapeutic perspectives to restore the cardiac metabolic flexibility by counteracting protein acetylation increase via pharmacological inhibition of KATs or replenishing strategies to restore NAD^+ levels and promote sirtuin deacetylase activity, as proposed by others (42–44).

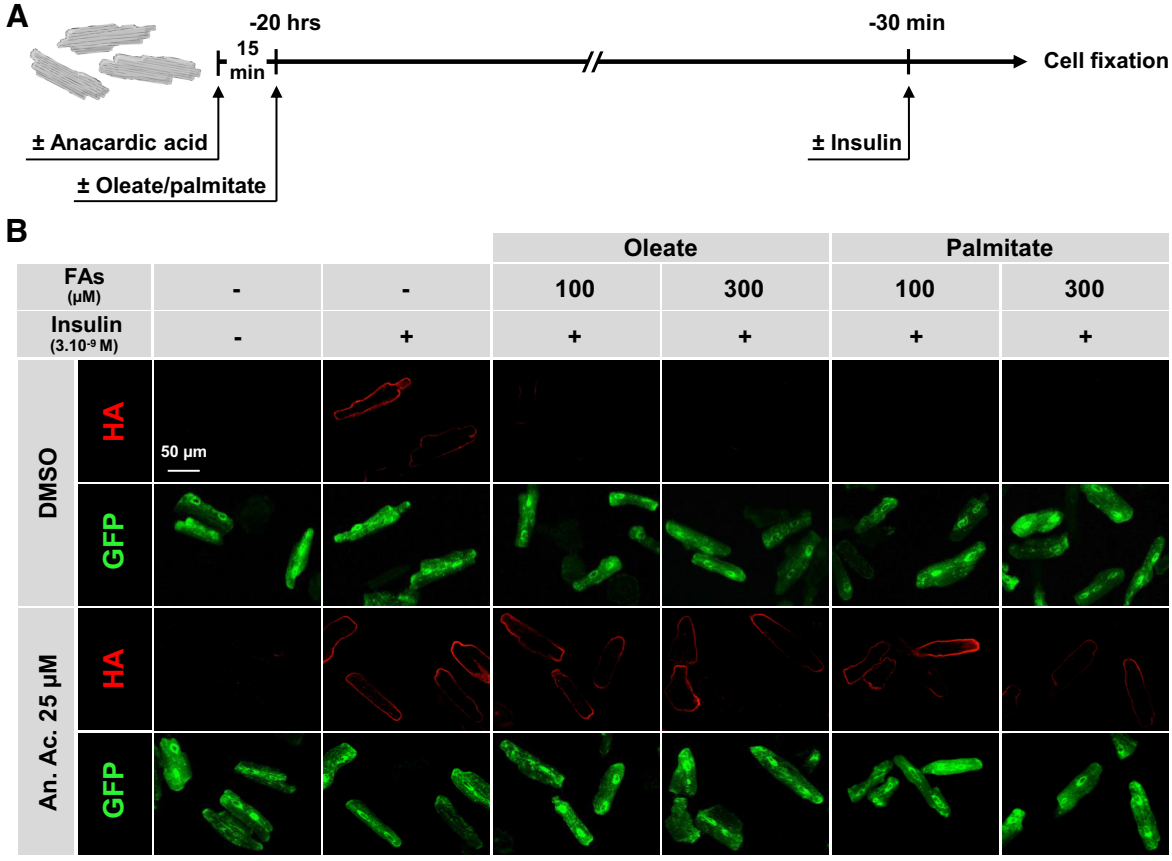
Nevertheless, such a broad strategy could be detrimental as it may globally affect cellular function, emphasizing the necessity of more targeted approaches. For this purpose, the identification of the acetylated proteins involved in GLUT4 translocation regulation and more particularly in the modulation of its exocytosis/endocytosis balance is of great interest. Indeed, our results show that regulation of glucose uptake by protein acetylation during cardiac metabolic overfueling affects mechanisms downstream of AS160 in the insulin and AMPK pathways. Moreover, this inhibition does not seem to be due to modifications in GLUT4 protein expression or acetylation level (20), highly suggesting a defect at the level of GLUT4 translocation to the plasma membrane. In line, the acetylation of the cytoskeletal component α-tubulin has been recently shown by our group to characterize the diabetic heart and to reduce GLUT4 translocation and glucose transport (24). Further research will be needed to *1*) identify other putative acetylated components involved in this regulation, *2*) determine the exact identity of the KAT(s) and/or sirtuin(s) controlling the acetylation level of these proteins under diabetic state, and *3*) understand the relationship between an excessive mitochondrial production of acetyl-CoA due to metabolic overload and the regulation of these cytoplasmic events. In addition, the rise in protein acetylation could also result from decreased deacetylase activity. Among other things, this could be due to a diminution of NAD^+ availability, the essential cofactor of sirtuin, reported to be depleted in metabolic overload circumstances (42, 44, 45).

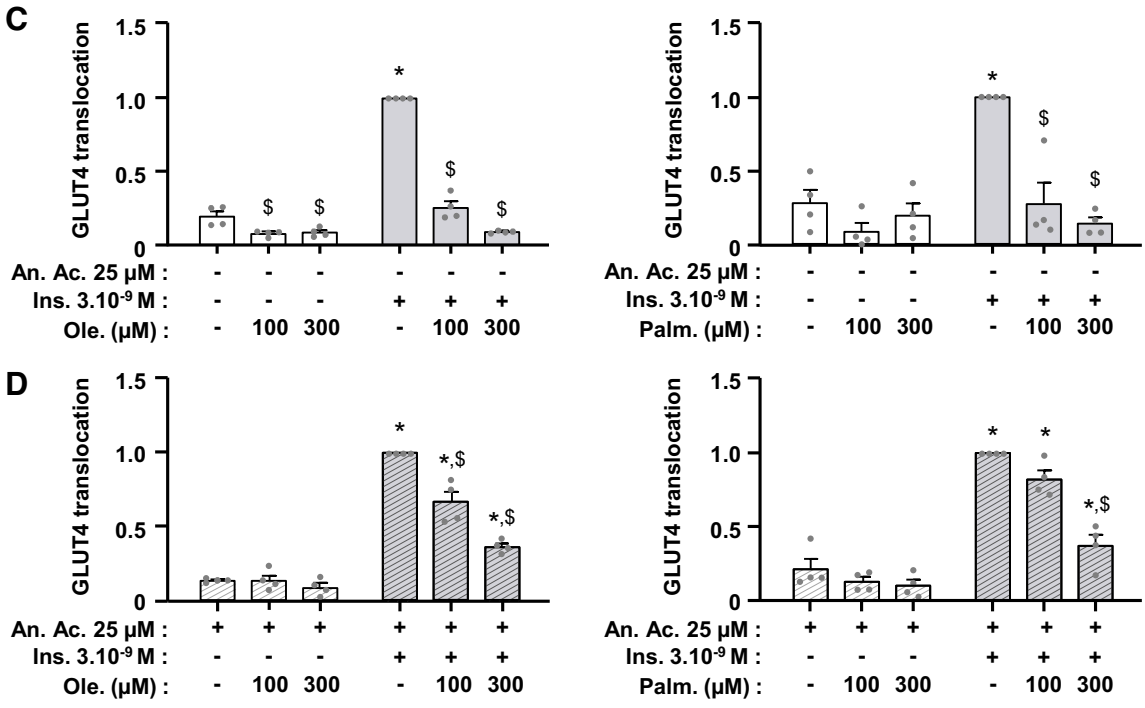
One of the major remaining interrogations is how universal is the regulation of GLUT4 translocation by protein acetylation. In addition to the heart, GLUT4 is expressed in skeletal muscle and adipose tissue where stimulation of its translocation by insulin is essential for glucose and metabolic homeostasis (46). Investigating if increased protein acetylation also participates in reduced GLUT4 translocation in these tissues is of significant importance. In addition, one may wonder whether stimulating glucose uptake in muscle by reducing protein acetylation may contribute to the regulation of overall glycemia, which would also be of great interest in the context of type 2 diabetes. Finally, GLUT4 translocation might not be the sole vesicular trafficking event altered by protein acetylation. It would be interesting to see if other metabolic transporters are similarly influenced by protein acetylation. For example, intracellular uptake of FAs largely depends on the relocalization at the plasma membrane of the transporter CD36 (47). Elucidating the impact of protein acetylation on CD36 trafficking could shed new light on the interactions between FA and glucose metabolism.

Figure 4. Oleate (Ole) and palmitate (Palm) induce a global rise in cardiac protein acetylation that mediates glucose uptake inhibition. *A*: experimental design to assess the role of protein acetylation in the inhibitory effects of oleate or palmitate on insulin (Ins)-stimulated glucose uptake in primary cultured adult rat cardiomyocytes. Representative immunoblots (*B*) of acetylated lysine (K-Ac) of proteins obtained from cardiomyocytes pretreated or not with 25 μM anacardic acid (An Ac) before oleate (*left*) or palmitate (*right*) treatment and their respective quantification (*C*). *D*: effect of oleate (*left*) or palmitate (*right*) on insulin-stimulated glucose uptake in cardiomyocytes pretreated or not with anacardic acid. Values are expressed as means ± SE of 4 independent experiments. Statistical analysis was conducted by two-way ANOVA. $*P \leq 0.05$, relative to respective insulin-unstimulated cells; $\$P \leq 0.05$, relative to respective FA-untreated cells in presence of insulin and in absence or presence of anacardic acid; $\#P \leq 0.05$, relative to respective anacardic acid-untreated cells. FA, fatty acid.

Limitations of the Study

The aim of this study was to investigate the role of oleate and palmitate on protein acetylation as part of the Randle cycle. We speculate that such mechanism could be exacerbated in a model of sustained elevated FAs and contribute to the profound metabolic reorganization in diabetic cardiomyopathy. However, our simplified model does not





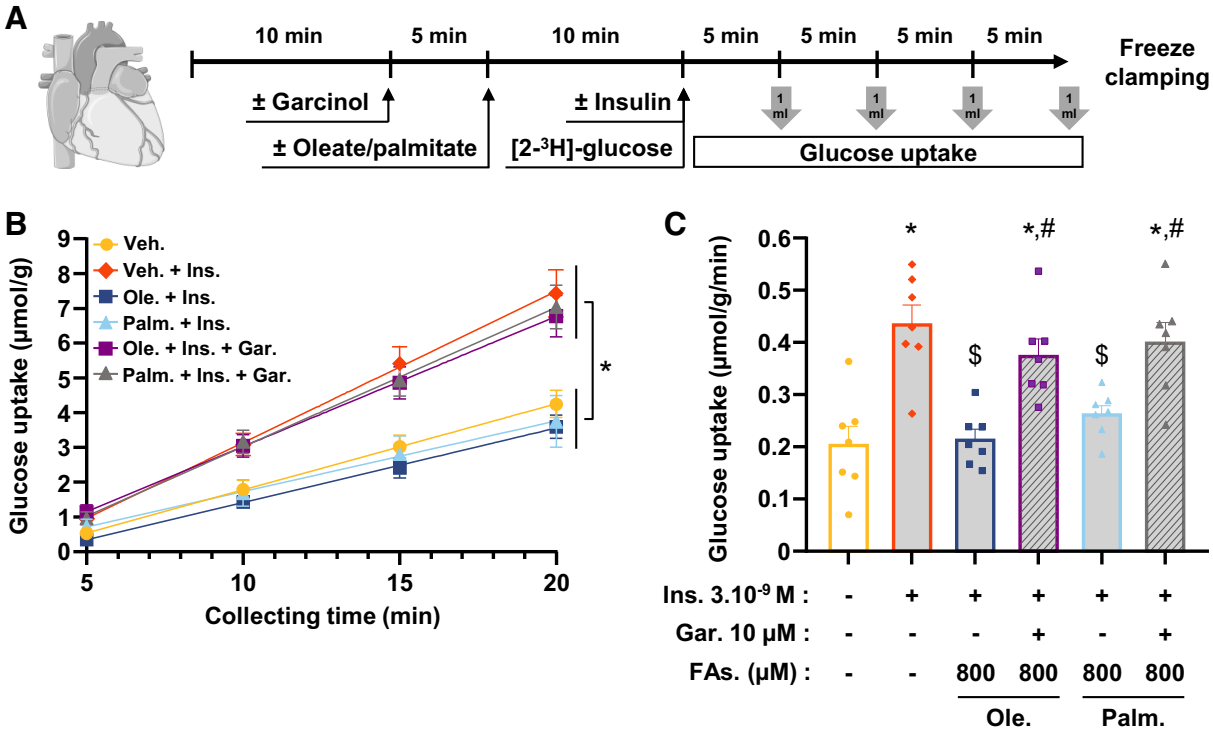


Figure 6. Oleate (Ole) and palmitate (Palm) inhibition of insulin (Ins)-stimulated glucose uptake in perfused hearts is prevented by KAT inhibitor. *A*: experimental design to assess the effect of oleate or palmitate on glucose uptake in perfused rat hearts. Cardiac glucose uptake measurement over time (*B*) and rate of glucose uptake corresponding to the slopes in *B* (*C*) in perfused hearts pretreated or not with 10 µM garcinol (Gar) before treatment with 800 µM oleate or 800 µM palmitate ($n = 7$ biological replicates in each group). Values are expressed as means ± SE. Statistical analysis was conducted by two-way ANOVA (*B*) or one-way ANOVA (*C*). *$P \leq 0.05$, relative to respective insulin-unstimulated hearts; $$P \leq 0.05$, relative to respective fatty acid (FA)-untreated hearts in presence of insulin and in absence or presence of garcinol; #$P \leq 0.05$, relative to garcinol-untreated hearts. Veh, vehicle (BSA).

address such a hypothesis, but it represents an important question that needs to be addressed by further studies. Moreover, we relied on generic KAT inhibitors as a means to highlight a role for protein acetylation as a whole, although such compounds may have unspecific effects. The use of relatively low doses of inhibitors and the fact that a similar effect is observed with two different inhibitors with their own specific action mechanism mitigate this risk and reinforce protein acetylation involvement in FA-mediated inhibition of glucose uptake.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Table S1: https://doi.org/10.6084/m9.figshare.19948532.

Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.19948610.

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DISCLOSURES

S.H. is Fonds National de la Recherche Scientifique Senior Research Associate. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

Figure 5. Reducing protein acetylation counteracts the inhibitory effects of oleate (Ole) and palmitate (Palm) on insulin (Ins)-stimulated GLUT4 translocation. *A*: experimental design to assess the role of protein acetylation in the inhibitory effects of oleate or palmitate on insulin-stimulated GLUT4 translocation in primary cultured adult rat cardiomyocytes. Representative images of fluorescence microscopy (*B*) and relative quantification of GLUT4 translocation expressed as the ratio of the red channel signal [hemagglutinin (HA):membrane] on the green channel signal [green fluorescent protein (GFP):total] in nonpermeabilized cardiomyocytes expressing HA-GLUT4-GFP protein in absence (*C*) or in presence (*D*) of 25 µM anacardic acid (An Ac) before treatment with oleate (100 or 300 µM, *left*) or palmitate (100 or 300 µM, *right*) ($n = 4$ biological replicates per group). Values are expressed as means ± SE relative to fatty acid (FA)-untreated cells in presence of insulin. Statistical analysis was conducted by two-way ANOVA. *$P \leq 0.05$, relative to respective insulin-unstimulated cells; $$P \leq 0.05$, relative to respective FA-untreated cells in presence of insulin and in absence or presence of anacardic acid.

AUTHOR CONTRIBUTIONS

M.D.L., E.R., A.G., C. Bouzin, S.H., C. Beauloye, L. Bertrand, and L. Bultot conceived and designed research; M.D.L., E.R., A.G., and L.B. performed experiments; M.D.L., E.R., A.G., C. Bouzin, S.H., C. Beauloye, L. Bertrand, and L. Bultot analyzed data; M.D.L., E.R., A.G., C. Bouzin, S.H., C. Beauloye, L. Bertrand, and L. Bultot interpreted results of experiments; M.D.L., E.R., A.G., L. Bertrand, and L. Bultot prepared figures; M.D.L., E.R., L. Bertrand, and L. Bultot drafted manuscript; M.D.L., E.R., A.G., C. Bouzin, S.H., C. Beauloye, L. Bertrand, and L. Bultot edited and revised manuscript; M.D.L., E.R., A.G., C. Bouzin, S.H., C. Beauloye, L. Bertrand, and L. Bultot approved final version of manuscript.

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