

Suppression of Rho-kinase 1 is responsible for insulin regulation of the AMPK/SREBP-1c pathway in skeletal muscle cells exposed to palmitate

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Abstract

Aims Clinical and experimental data suggest that early insulin therapy could reduce lipotoxicity in subjects and animal models with type 2 diabetes mellitus. However, the underlying mechanisms need to be clarified. Sterol regulatory element-binding protein 1c (SREBP-1c), which is negatively regulated by AMP-activated protein kinase (AMPK), plays a critical role in lipotoxicity and insulin resistance in skeletal muscle cells. Here, we investigated the effect and molecular mechanism of insulin intervention on the AMPK/SREBP-1c pathway in skeletal muscle cells with chronic exposure to palmitic acid (PA).

Methods Male C57BL/6 mice were fed with a high-fat diet for 12 weeks and were then treated with insulin, AMPK inhibitor, or metformin. L6 myotubes incubated with palmitic acid (PA) were treated with insulin or metformin.

Dominant-negative AMPK α 2 (DN-AMPK α 2) lentivirus, AMPK α 2 siRNA, or Rho-kinase 1 (ROCK1) siRNA were transfected into PA-treated L6 myotubes.

Results We found that the ability of PA to stimulate SREBP-1c and inhibit AMPK was reversed by insulin in L6 cells. Moreover, DN-AMPK α 2 lentivirus and AMPK α 2 siRNA were transfected into PA-treated L6 myotubes, and the decrease in SREBP-1c expression caused by insulin was blocked by AMPK inhibition independent of the phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K)/AKT pathway. The serine/threonine kinase Rho-kinase (ROCK) 1, a downstream effector of the small G protein RhoA, was activated by PA. Interestingly, knockdown of ROCK1 by siRNA blocked the downregulation of AMPK phosphorylation under PA-treated L6 myotubes, which indicated that ROCK1 mediated the effect of insulin action on AMPK.

Conclusions Our study indicated that insulin reduced lipotoxicity via ROCK1 and then improved AMPK/SREBP-1c signaling in skeletal muscle under PA-induced insulin resistance.

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The authors Sunyinyan Tang and Wenjun Wu contributed equally to this work.

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Keywords Rho-kinase 1 · Insulin · AMPK · SREBP-1c · Skeletal muscle

Introduction

Lipotoxicity, a well-accepted explanation for the development of obesity-associated insulin resistance, is characterized by elevated free fatty acid (FFA) levels in the plasma and excessive lipid accumulation in non-adipose tissue, namely liver and muscle. Clinical evidence has shown that early insulin therapy decreases FFA levels and improves dyslipidemia in patients with type 2

diabetes [1–3], and experimental data from diabetic animal models showed that insulin therapy reduces lipid content in the liver and muscle [4, 5]. However, the underlying molecular mechanism has not been completely elucidated.

Sterol regulatory element-binding protein 1c (SREBP-1c) is a key transcription factor that regulates *de novo* lipogenesis in insulin-sensitive tissues, including liver, muscle, and adipose tissue [6]. Aberrant activation of SREBP-1c leads to lipid accumulation, insulin receptor substrate 1 (IRS-1) suppression, and subsequent muscular insulin resistance [7, 8]. SREBP-1c activity is known to be upregulated by insulin and nutritional status [9] and downregulated by the cellular energy sensor AMP-activated protein kinase (AMPK) [10, 11]. Interestingly, our previous studies showed that insulin inhibits the increases in SREBP-1c expression induced by fatty acid in skeletal muscle *in vivo* and *in vitro* [12, 13], results that were similar to those of one study on macrophage cells in diabetes that showed the inhibitory effect of insulin on the increased SREBP-1c expression caused by high glucose [14], indicating that insulin might have different metabolic effects according to intracellular nutritional states.

The serine/threonine kinase Rho-kinase (ROCK), a downstream effector of the small G protein RhoA, exists as two isoforms, ROCK1 and ROCK2. ROCK1 is ubiquitously expressed in human and animal tissues, and ROCK2 expression is prominent in cardiac and brain tissues [15]. RhoA, activated by stimulation of tyrosine kinase and G-protein-coupled receptors (GPCRs), interacts with the Rho-binding domain of ROCK and alters the conformation of ROCK, resulting in ROCK action on downstream molecules. ROCK1 deletion can have a negative or positive effect on insulin signaling in skeletal muscle [16–18]. In addition, studies in animals and humans showed that ROCK is activated in metabolic syndrome, while the inhibition of ROCK ameliorates metabolic disorders [19, 20]. Further studies revealed that AMPK is the effector of ROCK in metabolic syndrome and aortic endothelial dysfunction in diabetic animal models [21–23], but the effect of insulin action on ROCK is unknown.

In the present study, we investigated the effects and molecular mechanisms of insulin intervention on the AMPK/SREBP-1c pathway in skeletal muscle cells with a lipid surplus. Our results demonstrated that insulin increased AMPK α phosphorylation and decreased SREBP-1c expression in palmitate (PA)-treated skeletal muscle cells *in vivo* and *in vitro*, which was mediated by the suppression of ROCK1, which indicated that an interaction between the insulin signaling and AMPK appeared to play an important role in early insulin therapy of type 2 diabetes.

Methods

Antibodies

Antibodies against AMPK α 1/2, phospho-AMPK α (Thr172), AKT, phospho-AKT (Ser473), acetyl-CoA carboxylase (ACC), phospho-acetyl-CoA carboxylase (Ser79)(p-ACC), and Rho-kinase 1 (ROCK1) were purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-SREBP-1 antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against IRS-1, phospho-IRS-1 (Tyr608/612), and GAPDH were from EMD Millipore (Billerica, MA, USA).

Animals and intervention

Male C57BL/6 mice at 6 weeks of age were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, China) and were maintained on a 12-h light–dark cycle (temperature 23 ± 1 °C, humidity 45–65%) and fed on a standard chow diet (70% carbohydrates, 10% fat, and 20% protein; CON) or a high-fat diet (20% carbohydrates, 60% fat, and 20% protein; HFD) for 12 weeks. The HFD mice were then randomized into 4 groups (5 per group): (1) HFD control group, injected with phosphate-buffered saline (PBS) as a vehicle control; (2) metformin treatment group (HFD+Met), administered metformin (Sigma-Aldrich, St. Louis, MI, USA) by gavage at a dosage of $250 \text{ mg kg}^{-1} \text{ day}^{-1}$; (3) insulin treatment group (HFD+Ins), treated with Glargine (Sanofi-Aventis, France) by subcutaneous injection at a dosage of 0.5 IU; and (4) insulin and compound C treatment group (HFD+Ins+CC), treated with insulin and compound C (Sigma-Aldrich, St Louis, MI, USA) by intraperitoneal injection at a dosage of $20 \text{ mg kg}^{-1} \text{ day}^{-1}$. The medication dosages were adjusted to maintain blood glucose (BG) close to that of the CON mice. BG was measured in the tail vein with a glucometer (Accu-Check Active, Roche diagnostic, Germany) at 10 am. After 2 weeks of intervention, mice were deprived of food for 8 h and then killed for tissue collection. All animal procedures were performed according to the National Institutes of Health guidelines and approved by the animal care committee of Drum Tower Hospital affiliated with Nanjing University Medical School (Nanjing, China).

Cell culture

L6 cells were purchased from the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, vol./vol.), 100 U/ml

penicillin, and 100 µg/ml streptomycin. Cells were differentiated into myotubes in DMEM with 2% FBS for 5–7 days with medium replacement every day. The cells were then incubated in serum-free medium for 8 h and treated with 0.5 mmol/l PA for 24 h. After incubation with PA, cells were treated with 100 nmol/l insulin for a further 12 h. To determine whether AMPK or AKT was involved in the effect of insulin, cells were pretreated with AMPK inhibitor compound C at 10 µmol/l or with phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K) inhibitor wortmannin at 1 µmol/l (Sigma-Aldrich, St. Louis, MI, USA) for 30 min prior to PA treatment. Cell lysates were collected for western blotting analysis.

Glucose uptake assay

L6 cells were treated with PA for 24 h and 100 nmol/l insulin for a further 12 h. The medium was then replaced with low-glucose and serum-free DMEM containing 100 µmol/l 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-2-deoxy-D-glucose (2-NBDG, Cayman, Ann Arbor, MI, USA). After incubation for 1 h at 37 °C in the dark, cells were collected, and the fluorescence intensity was measured at an excitation of 485 nm and emission of 520 nm using a FACS Calibur flow cytometer (BD Bioscience).

Recombinant lentivirus infection

A recombinant lentivirus encoding a rat c-Myc-tagged dominant-negative AMPK α 2 (DN-AMPK α 2; aspartic acid was substituted for alanine¹⁵⁹) was purchased from GeneChem (Shanghai, China). L6 cells grown in one well of a 6-well plate were infected with 50 MOI of DN-AMPK α 2 lentiviruses or with GFP lentiviruses as a control. The culture medium was changed 6 h later. At 48 h, protein extracts were harvested for detection by the following methods.

RNA interference

AMPK α 2 and ROCK1 gene expression were silenced with small interfering RNA (siRNA). The siRNA sequence for targeting AMPK α 2 or ROCK1 (GenePharma, Shanghai, China) was 5'-CCACUCUCCUGAUGCAUATT-3' or 5'-GGGUAACUCAUCUGGUAATT-3', respectively. The siRNA sequence used as a negative control (siRNA-NULL) was 5'-UUCUCCGAACGUGUCACGUTT-3'. L6 myoblasts were transfected with siRNA (100 nM) via Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, cells were treated with 0.5 mmol/l PA for an additional 24 h, and then, cells were harvested for western blotting analysis.

Western blotting

The treated cells were lysed in ice-cold cell lysis buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The soleus muscle (approximately 200 mg) was homogenized in ice-cold buffer. Protein concentrations were determined by the bicinchoninic acid (BCA) assay. Protein lysates were boiled for 5 min and dissolved in Laemmli buffer. 20 mg of protein per lane was separated by SDS-PAGE, then transferred onto polyvinylidene fluoride membranes, blocked with 5% nonfat milk (wt./vol.), washed with Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with the appropriate primary antibodies overnight at 4 °C. After three washes with TBST, membranes were incubated with the appropriate HRP-conjugated secondary antibodies for 2 h at room temperature, followed by detection with enhanced chemiluminescence (EMD, Millipore). Band intensities were quantified with Quantity One (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The data are expressed as the mean \pm SE. The differences among groups were analyzed by one-way ANOVA with least significant difference (LSD) or Dunnett T3 post hoc comparison analysis, as appropriate. Values of $p < 0.05$ were considered statistically significant.

Results

The ability of PA to stimulate SREBP-1c and inhibit AMPK in L6 cells is reversed by insulin

First, the effects of insulin on the AMPK/SREBP-1c pathway and insulin signaling were studied. As shown in Fig. 1a, insulin stimulated 2-NBDG uptake in control cells, and PA decreased 2-NBDG uptake, which was upregulated by insulin. To determine the effect of PA and insulin on insulin signaling and the AMPK/SREBP-1c pathway in vitro, p-IRS-1 (Tyr608/612), p-AKT (Ser473), mTOR, SREBP-1c, and fatty acid synthase (FAS) were measured. In control cells, insulin stimulated the phosphorylation of IRS-1 (Tyr608/612) and AKT (Ser473) and the expression of mTOR (Fig. 1c) and increased the expression of SREBP-1c and its target molecule FAS (Fig. 1b). In cells exposed to PA, p-IRS-1 (Tyr608/612) and p-AKT (Ser473) were reduced (Fig. 1c). Meanwhile, AMPK α 2 phosphorylation and phospho-acetyl-CoA carboxylase were decreased (Fig. 1d), and SREBP-1c was significantly increased (Fig. 1b). However, the ability of PA to stimulate SREBP-1c and inhibit AMPK was reversed by insulin, and p-IRS-1 (Tyr608/612) and p-AKT (Ser473) increased in L6 cells after insulin intervention.

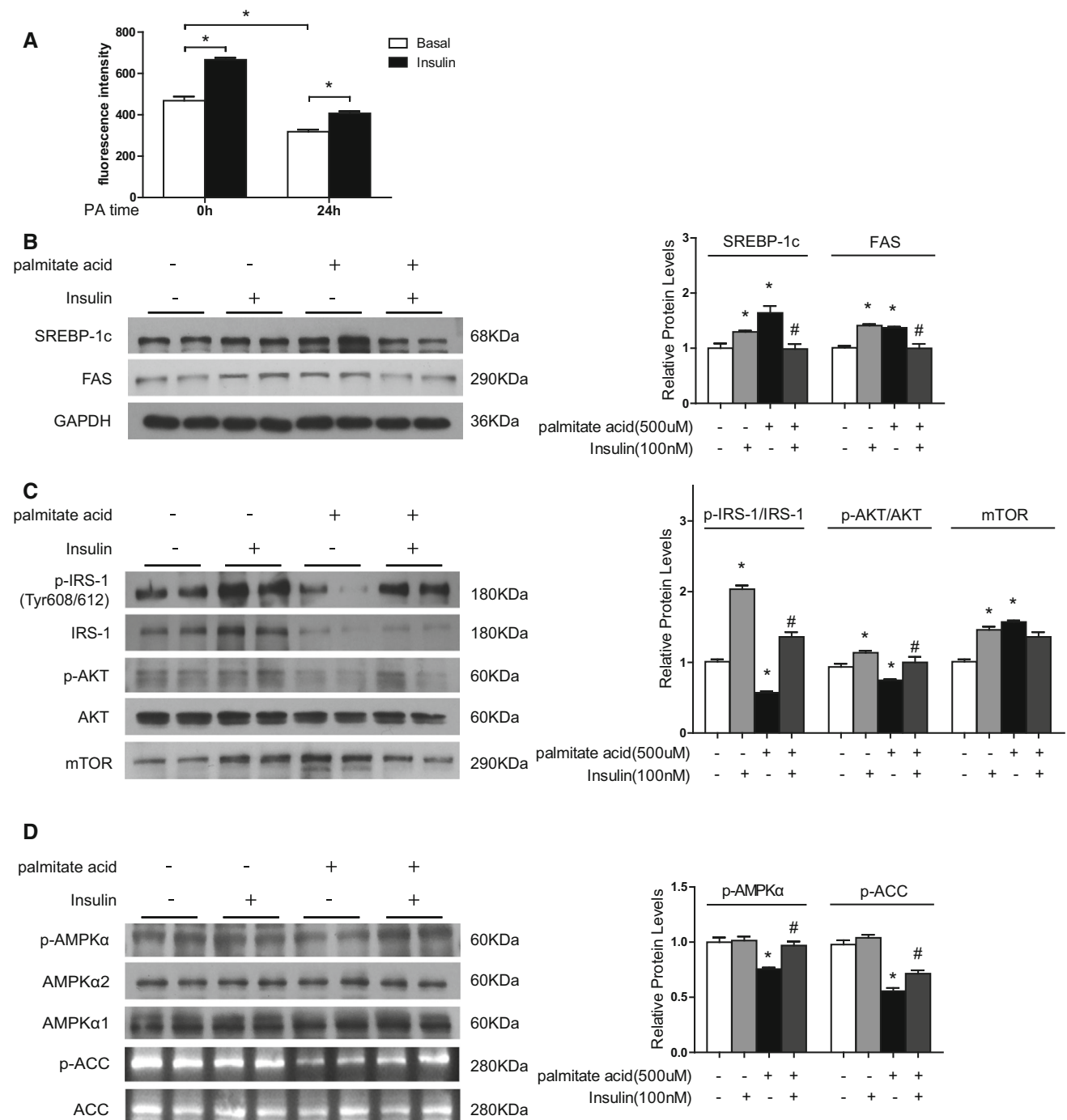


Fig. 1 Insulin promoted glucose uptake, and the ability of PA to stimulate SREBP-1c and inhibit AMPK was reversed by insulin in L6 cells. **a** 2-NBDG uptake was measured following PA treatment. **b** The SREBP-1c and FAS protein expressions in L6 cells were determined by western blotting incubated with PA either in the absence or

presence of insulin. **c** Insulin signaling protein expressions incubated with PA and insulin. **d** p-AMPK α /AMPK α 2 and p-ACC expressions. Data are expressed as the mean \pm SE of three independent experiments. The significance between groups is presented as indicated; * $p < 0.05$ versus control cells. # $p < 0.05$ versus PA-treated cells

The decrease in SREBP-1c expression caused by insulin is blocked by AMPK inhibition independent of the PI3K/AKT pathway

Next, the effects of insulin intervention on SREBP-1c were studied by using the AMPK inhibitor compound C or the

PI3K inhibitor wortmannin. As shown in Fig. 2, insulin decreased PA-induced SREBP-1c, and this effect was blocked by AMPK inhibitor but not by PI3K inhibitor, indicating that insulin decreased SREBP-1c expression through AMPK activation independent of the PI3K/AKT pathway in L6 cells with chronic PA elevation. These

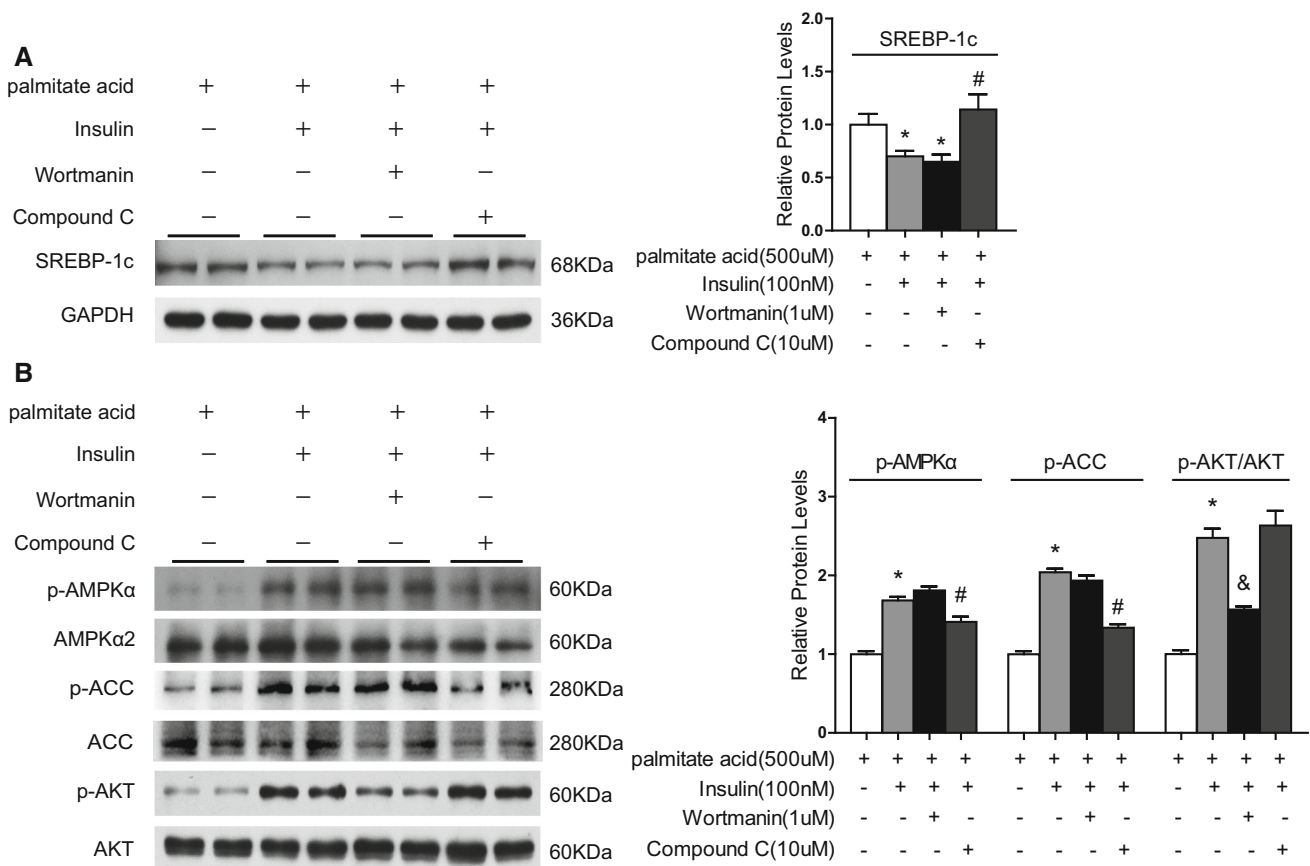


Fig. 2 SREBP-1c expression decreased by insulin was blocked by AMPK inhibition independent of the PI3K/AKT pathway. **a** The SREBP-1c protein expressions in L6 cells were determined by western blotting. **b** The p-AMPK α , p-AKT/AKT and p-ACC expressions were determined by western blotting. Data are expressed

as the mean \pm SE of 3 independent experiments. The significance between groups is presented as indicated; * $p < 0.05$ versus PA-treated cells. # $p < 0.05$, insulin combined with compound C-treated cells versus insulin-treated cells. & $p < 0.05$, insulin combined with wortmannin-treated cells versus insulin-treated cells

results suggest that AMPK is involved in insulin's down-regulation of SREBP-1c in skeletal muscle cells incubated with PA.

Insulin, via activation of AMPK α 2, alleviates the increase in SREBP-1c induced by fatty acid in skeletal muscle in vivo and in vitro

High-fat diet (HFD)-induced diabetic mice were treated with medical interventions for 2 weeks. Compared to chow diet mice, the HFD mice had a marked increase in random BG (blood glucose) levels. After 2 weeks of insulin administration, their hyperglycemia was restored to near normal. Compared with insulin treatment, insulin combined with compound C had no effect on hyperglycemia or body weight (Fig. 3a, b).

As shown in Fig. 3c and Fig. 1S, AMPK α 2 and AKT phosphorylation decreased and SREBP-1c and ROCK1 increased in skeletal muscle of HFD mice compared with those in the chow diet mice. By contrast, insulin treatment increased AMPK α 2 and AKT phosphorylation and

downregulated SREBP-1c compared with those in the HFD mice. Insulin co-treatment with compound C blocked the effects of insulin on increased AMPK α 2 phosphorylation and suppressed SREBP-1c expression (Fig. 3c). To further investigate the role of AMPK in insulin-reduced SREBP-1c expression in an independent setting, we used siRNA targeting AMPK α 2. As shown in Fig. 3d, compared with transfection with siRNA-NULL, AMPK α 2 knockdown mitigated the insulin-induced decrease in SREBP-1c protein under the condition of chronic PA treatment. We used DN-AMPK α 2 to downregulate AMPK α 2 activation and showed that the downregulation of SREBP-1c by insulin was restored in L6 cells cultured with PA (Fig. 3e). These results indicated that insulin decreased SREBP-1c expression through AMPK activation in L6 cells with chronic PA elevation.

ROCK1 mediates the effect of insulin on AMPK

We determined whether ROCK1 mediated the effect of insulin action on AMPK in PA-treated skeletal muscle

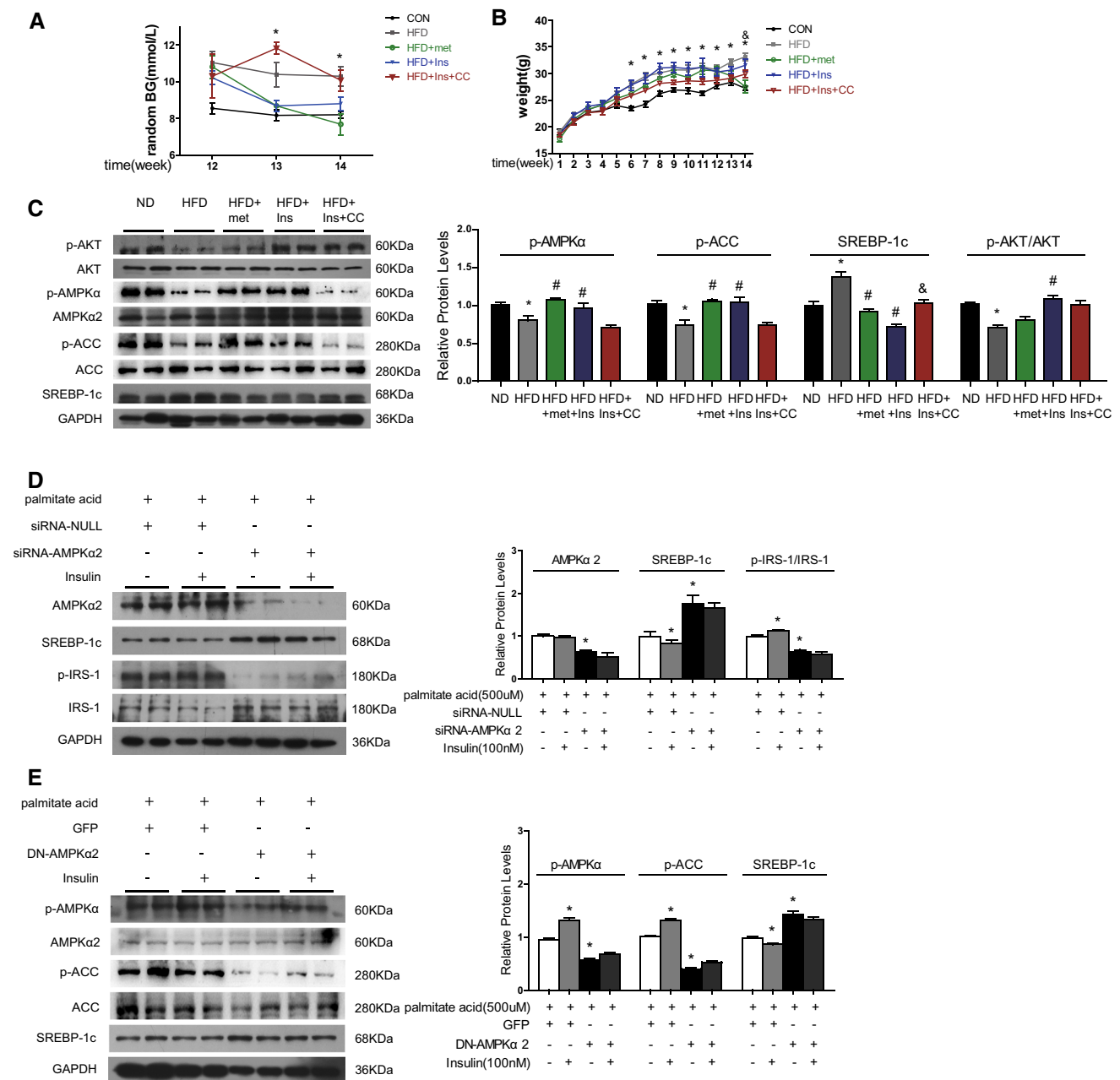


Fig. 3 Insulin, via activation of AMPK α 2, alleviated the increment of SREBP-1c induced by fatty acid in skeletal muscle *in vivo* and *in vitro*. Mice were fed normal chow diet (ND, $n = 6$), high-fat diet ($n = 6$), HFD supplemented with metformin (HFD+met, $n = 6$), HFD supplemented with insulin (HFD+Ins), or HFD supplemented with insulin combined with Compound C (HFD+Ins+CC). Mice were initially fed on a HFD for 12 weeks, followed by HFD plus medical treatment for additional 2 weeks. The body weight (**a**) and non-fasting BG (**b**) of all mice were monitored during the intervention study. Values are the mean \pm SE ($n = 6$). **c** The muscle p-AMPK α , SREBP-1c protein expression was determined by western blotting after insulin, metformin, or insulin combined Compound C treatment

in HFD-fed mice. SREBP-1c expression decreased by insulin was blocked by AMPK inhibitor. The significance between groups is presented as indicated; * $p < 0.05$ versus ND. # $p < 0.05$ versus HFD. & $p < 0.05$ versus HFD+Ins. **d** Downregulation of AMPK activity by AMPK α 2 siRNA attenuated the impact of insulin on the inhibition of SREBP-1c in PA-treated L6 myotubes. **e** DN-AMPK α 2 lentivirus blocked the effect of insulin on the inhibition of SREBP-1c. The p-AMPK α , p-AKT/AKT, p-IRS-1/IRS-1, SREBP-1c protein expressions in L6 cells were determined by western blotting. Data are expressed as the mean \pm SE of three independent experiments. The significance between groups is presented as indicated; * $p < 0.05$ versus control cells

cells. As shown in Fig. 4, compared with the control, PA treatment increased ROCK1 expression, which was inhibited by additional insulin treatment. Furthermore,

knockdown of ROCK1 by siRNA blocked the PA-induced downregulation of AMPK and decreased the expression of SREBP-1c, AKT, and FAS. Taken together, these results

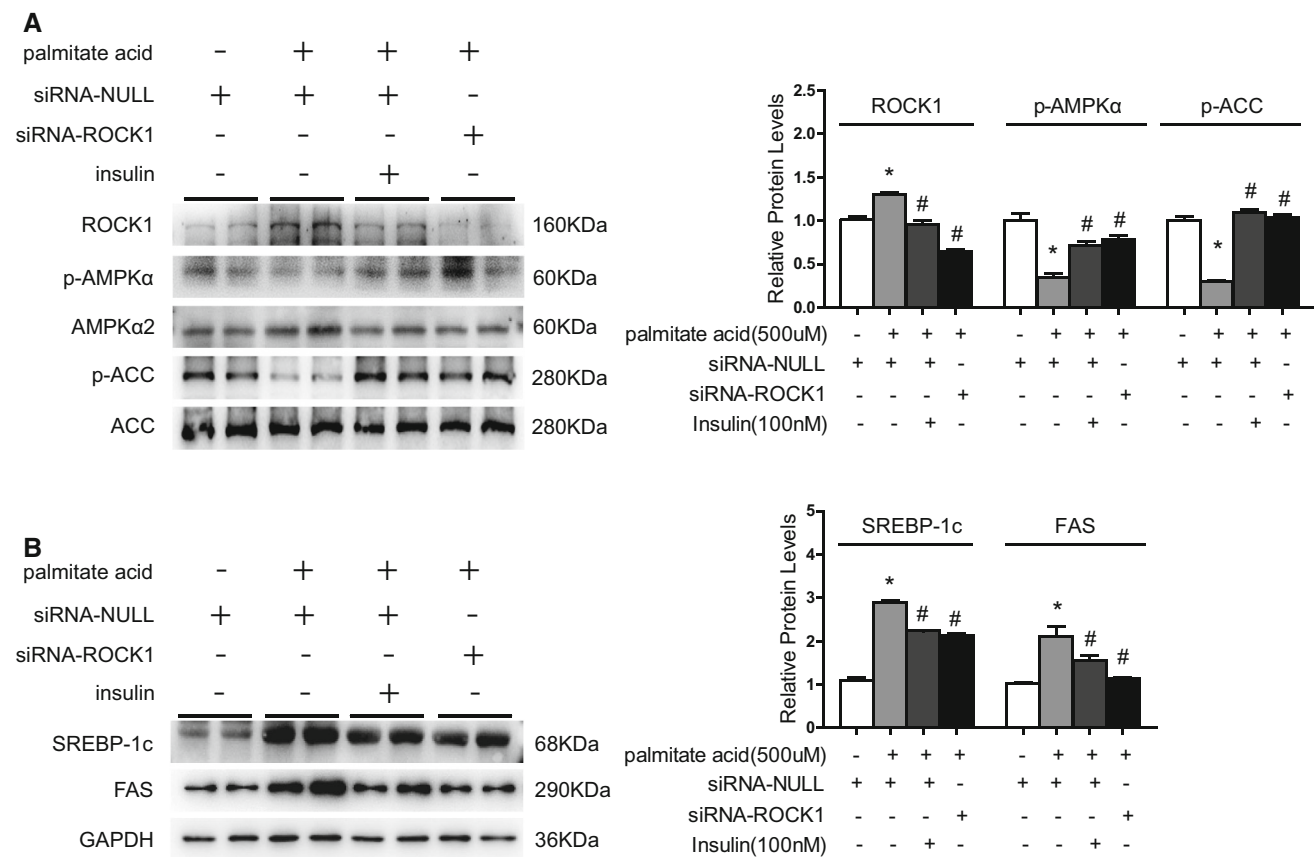


Fig. 4 Knockdown of ROCK1 by siRNA blocked the downregulation of PA-induced SREBP-1c and FAS levels. **a** Protein levels of ROCK1, p-AMPK α , and p-ACC in L6 cells were determined by western blotting. **b** SREBP-1c and FAS protein expressions were

determined by western blotting. Data are expressed as the mean \pm SE of three independent experiments. The significance between groups is presented as indicated; * $p < 0.05$ versus control cells. # $p < 0.05$ versus PA-treated cells

indicate that PA stimulated ROCK1, which led to metabolic disorder by upregulation of fatty acid synthesis, and that the inhibition of ROCK1 by insulin may improve the action of the AMPK/SREBP-1c pathway to downregulate lipid accumulation in PA-treated skeletal muscle cells.

Discussion

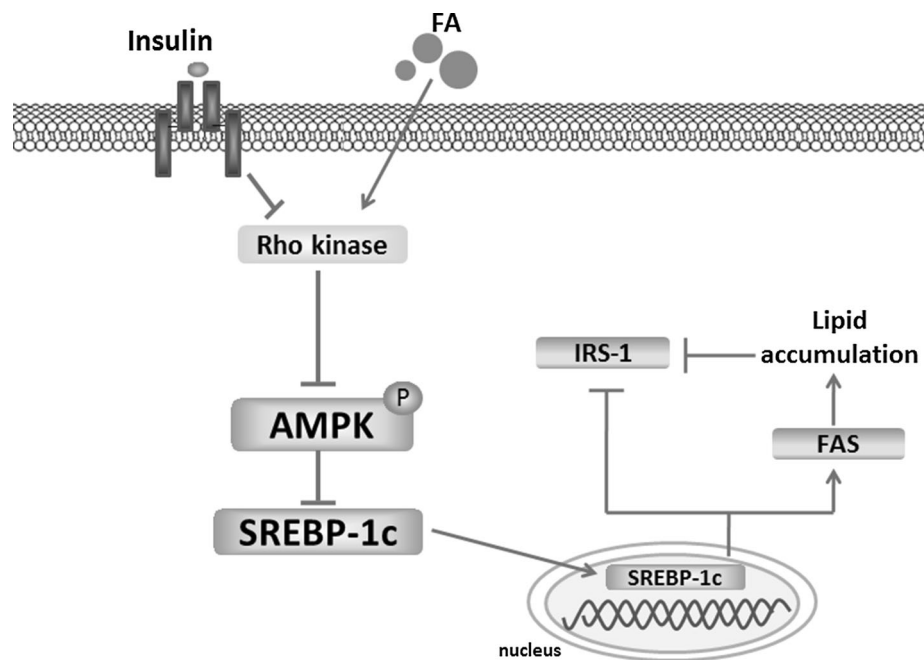
The major finding of the current study is that insulin, via activation of AMPK α 2, alleviated the increase in SREBP-1c induced by fatty acids in skeletal muscle in vivo and in vitro. Moreover, the suppression of ROCK1 potentially accounted for the molecular mechanism of insulin regulation of the AMPK/SREBP-1c pathway.

Chronic exposure to excess nutrients, especially saturated fatty acids, leads to insulin resistance in skeletal muscle [24]. SREBP-1c plays a role in the molecular mechanism of insulin resistance by causing lipotoxicity in the case of chronic PA elevation. Our recent data revealed that SREBP-1c could directly suppress insulin receptor substrate 1 (IRS-1) expression and the subsequent insulin

signaling pathway in PA-treated skeletal muscle cells [8]. Thus, SREBP-1c plays a critical role in skeletal muscle lipotoxicity.

Insulin's stimulation of SREBP-1c is PI3K/AKT dependent [25]. Inhibition of PI3K by chemical inhibitors or expression of a dominant-negative AKT inhibits ER-to-Golgi transport of SREBP-1c and proteolytic activation. Consistent with this, insulin stimulates ER-to-Golgi transport of SREBP-1c by promoting its phosphorylation and association with COPII vesicles [26]. The mammalian target of rapamycin (mTOR) kinase is the major PI3K/AKT downstream effector. Rapamycin, the mTOR inhibitor, potently inhibits insulin-stimulated SREBP activation in primary rat hepatocytes [27]. Our data show that insulin stimulated AKT phosphorylation and mTOR expression, which promoted SREBP-1c and its target molecule FAS in control cells. However, a somewhat surprising finding in our study was that insulin inhibited SREBP-1c expression in skeletal muscle cells with lipid surplus, which was different from the SREBP-1c response to insulin in physiological states. Further in vivo and in vitro experiments showed that the decrease in SREBP-1c

Fig. 5 Research model: Suppression of ROCK1 was responsible for insulin-dependent regulation of the AMPK/SREBP-1c pathway in skeletal muscle cells exposed to PA



expression due to insulin was blocked by AMPK inhibition but not by PI3K inhibition, indicating that AMPK was involved in the insulin inhibition of SREBP-1c expression in skeletal muscle cells incubated with PA.

AMPK is a serine-threonine kinase and a key metabolic regulator [19]. In the physiological state, insulin decreases AMPK activity by downregulating phosphorylation on Thr172 of its α -subunit. The mechanism involved in the AMPK inactivation is the upregulation of the AKT/mTOR pathway [28]. Activation of AMPK enhances insulin sensitivity in a variety of tissues, including heart, liver, adipose tissue, and skeletal muscle [29, 30], whereas loss of AMPK contributes to insulin resistance [31]. With regard to fatty acid metabolism in skeletal muscle, AMPK reduces fatty acid synthesis by inhibiting SREBP-1c [11]. Experimental data from diabetic animal models have shown that insulin therapy reduces lipid content in liver and muscle by downregulating SREBP-1c [4, 5]. Our data show that AMPK α 2 phosphorylation was decreased by PA treatment and that insulin intervention increased p-AMPK α and decreased SREBP-1c protein expression. Moreover, the decrease in SREBP-1c by insulin was blocked by AMPK inhibition in skeletal muscle cells incubated with PA, indicating that insulin decreased SREBP-1c expression through AMPK activation under lipid surplus conditions, which was different from insulin's effect on AMPK inhibition in the physiological state. However, the effect and mechanism of insulin treatment on the AMPK pathway in skeletal muscle with PA elevation needs to be explored.

Environmental factors regulate intracellular signal transduction through Rho-kinase, leading to cell function

and metabolic changes [32]. Studies in animals and humans have shown that ROCK is activated in metabolic syndrome, while the inhibition of ROCK ameliorates metabolic disorders by activation of the AMPK pathway [19, 20]. Lipid-induced insulin resistance in muscle occurs through activation of the RhoA/Rho-kinase signaling pathway [33], implying a crucial role of the RhoA/Rho-kinase pathway in skeletal muscle, in which it mediates lipid-induced insulin resistance in obese mice. Consistent with these studies, we also found that ROCK1 expression was increased in L6 myotubes under PA-induced insulin-resistant conditions. Furthermore, knockdown of ROCK1 by siRNA increased AMPK activity and blocked the PA-induced upregulation of fatty acid synthesis-related protein SREBP-1c and FAS. The ROCK inhibitor fasudil improves IRS-1-dependent insulin signaling in skeletal muscle cells of hyperinsulinemic Zucker rats [34]. Rho-kinase inhibits AMPK, with a resultant reduction in whole-body energy consumption and dyslipidemia [21]. Rho-kinase inhibition increases AMPK phosphorylation and increases energy consumption, resulting in the amelioration of lipid profiles in *n/i/eNOS*^{-/-} mice [22]. These results indicate that the lipid-lowering effects of Rho-kinase inhibition are mediated by AMPK phosphorylation, suggesting that Rho-kinase is also an upstream effector of AMPK and an important therapeutic target of metabolic disorders.

Insulin enhances the vasodilator capacity of VSMCs by inactivating the small GTPase RhoA and its target ROCK [35]. Unlike the other effects of insulin, which are mediated by the PI3K/AKT signaling pathway [36], insulin's effect on Rho-kinase inhibition does not appear to involve

PI3K, as wortmannin does not prevent insulin-mediated Rho-kinase inactivation even though it completely abolishes insulin-mediated PI3K/AKT activation [35]. Furthermore, treatment with sodium nitroprusside (SNP), a nitric oxide (NO) donor, mimics the effect of insulin on Rho-kinase inactivation, indicating that insulin inhibits Rho-kinase activity via nitric oxide synthase (NOS) signaling [35, 37]. Our results seem to suggest that the decrease in SREBP-1c expression caused by insulin was blocked by AMPK inhibition independent of the PI3K/AKT pathway, and insulin attenuated the PA-induced upregulation of fatty acid synthesis-related protein SREBP-1c and FAS by inactivating ROCK1 expression. Why wortmannin failed to block insulin's effect on SREBP-1c in skeletal muscle in a PA-induced insulin-resistant state is not clear [35]. And the activation of AMPK promoted GLUT4 location to the cell membrane to increase glucose uptake [29]. Thus, the present study indicated that activation of AMPK partly mediated such effects in addition to PI3K/AKT pathway in PA-induced insulin resistance. Further studies are needed to clearly establish the mechanism by which insulin inactivates Rho-kinase and how this inactivation leads to the downregulation of SREBP-1c. Nonetheless, these results, though indirect, support our hypothesis: Suppression of ROCK1 was responsible for insulin-dependent regulation of the AMPK/SREBP-1c pathway in skeletal muscle cells exposed to PA (Fig. 5).

In conclusion, our study revealed that insulin reduced lipotoxicity via ROCK1 and then improved AMPK/SREBP-1c signaling in skeletal muscle under PA-induced insulin resistance. An interaction between the insulin signaling and AMPK appears to play a role in early insulin therapy of type 2 diabetes, which implies that insulin combined with an AMPK activator could serve as a more effective treatment for achieving complete remission of type 2 diabetes.

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Author contributions Yan Bi and Dalong Zhu contributed to the study design, data interpretation, and final approval of the version to be published. Sunyinyan Tang and Wenjun Wu wrote the main

manuscript text and contributed to the acquisition of data and approval of the final version. Wenjuan Tang, Zhijuan Ge, and Ting Hong prepared Figures 1–4 and contributed to acquisition of data. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest Sunyinyan Tang, Wenjun Wu, Wenjuan Tang, Zhijuan Ge, Ting Hong, Dalong Zhu, and Yan Bi declare that they have no competing financial interests.

Ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Statement of human and animal rights All animal procedures were performed according to the National Institutes of Health guidelines and approved by the animal care committee of Drum Tower Hospital affiliated with Nanjing University Medical School (Nanjing, China).

Informed consent Our study is not clinical trial, so there is no informed consent obtained from individual participants included in the study.

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