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ORIGINAL ARTICLE

Overactivation of NF-κB impairs insulin sensitivity and mediates palmitate-induced insulin resistance in C2C12 skeletal muscle cells

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Abstract Lipid-induced insulin resistance is associated with inflammatory state in epidemiological studies. However, it is still unclear whether the activation of NF-κB, a pivotal transcription factor of inflammation, plays a crucial role in mediating skeletal muscle insulin resistance. This study addressed what was the role of NF-κB in lipid-induced insulin resistance and whether NF-κB activation was sufficient to cause insulin resistance in C2C12 myotubes. A 16 h exposure of myotubes to palmitate reduced net insulin-stimulated glucose uptake by 48%, GLUT4 translocation by 52%, Akt phosphorylation by 54%, induced a 1.8-fold increase in insulin-stimulated insulin receptor substrate (IRS) phosphorylation, and doubled NF-κB activation. Myotubes transfected with NF-κB p65 siRNA for 24 h and followed by a treatment with palmitate for 16 h efficiently blocked NF-κB activation, and prevented the detrimental effects of palmitate on the metabolic actions of insulin. Transfection of myotubes with I-κBα siRNA for 24 h also led to a twofold induction of NF-κB activation, and reduced net insulin-stimulated glucose uptake by 30%, GLUT4 translocation by 35%, Akt phosphorylation by 31%, induced a 0.7-fold increase in insulin-stimulated IRS phosphorylation. These findings suggest that NF-κB overexpression per se is sufficient to impair insulin sensitivity and palmitate-induced insulin resistance is mediated by NF-κB in skeletal muscle cells.

Keywords Small Interfering RNA · Skeletal muscle · Insulin resistance · Palmitate · NF-kappa B · I-kappa B

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Introduction

Insulin resistance is decreased the response of the peripheral tissues to insulin action. It plays a primary role in the development of type 2 diabetes and is a characteristic feature of other health disorders including obesity, dyslipidemias, hypertension, cardiovascular disease, and metabolic syndrome. Chronic elevation in plasma-free fatty acid (FFA) levels is commonly associated with insulin resistance [1] and often coexists with obesity and type 2 diabete [2, 3]. And acute elevations in plasma FFA levels has also been proved to decrease insulin-stimulated glucose uptake [4].

The mechanisms by which elevated levels of FFA impairs insulin action are not well understood. Growing evidence links a subclinical inflammatory state to pathogenesis of insulin resistance [5]. Epidemiologists have consistently found elevations in markers and potential mediators of inflammation in diabetics [6, 7], suggesting inflammation predicts the development of insulin resistance.

Administration of salicylates to diabetics is observed to result in an insulin-sparing effect [8, 9]. The molecular target of salicylates is inhibitor κB kinase β(IKKβ), the enzyme lies upstream of the transcription factor NF-κB. NF-κB is regarded as the central mediator of gene regulation in the inflammatory response [10, 11]. NF-κB family proteins are characterized by the presence of a highly

conserved Rel homology domain, which is implicated in dimerization of NF-κB, DNA binding, and association with the inhibitory proteins IκBs [12]. Interactions between members of the NF-κB family result in the formation of dimers (homodimers or heterodimers) and the most commonly occurring heterodimer is p50/65 [13]. In basal states, IκBα forms a complex with p50/p65. After stimulation, IκBα is phosphorylated by IKK complexes, ubiquitinated, and degraded. The free NF-κB complex then translocates into the nucleus where it activates target gene transcription. IκBs also plays an important role in termination of NF-κB activation. Newly synthesized IκBα enters the nucleus and binds NF-κB, thereby enhancing its dissociation from the DNA [12].

Lipid-induced insulin resistance in muscles might be associated with IKKβ/IκBα/NF-κB pathway [14]. The hypothesis of a causative link between this inflammatory pathway and insulin resistance is proposed based on evidence that blockade of IKKβ/IκBα/NF-κB passway prevents detrimental effects of lipid on the metabolic actions of insulin [15, 16]. However, some doubt has been cast on this hypothesis. Polkinghorne et al. [17] separately overexpressed the p65 subunit of NF-κB and IKKβ in single muscles of rats for 1 week using in vivo electrotransfer but concluded that activation of the IKKβ/NF-κB pathway did not seem to be an important local mediator of insulin resistance. Even in studies supporting some causative relationship exists between IKKβ/NF-κB pathway and skeletal muscle insulin resistance, the role of NF-κB is still ambiguous. Studies of NF-κB inhibition suggested NF-κB was key element in disease pathogenesis whereby specifically inhibiting NF-κB improved insulin signaling proteins and ameliorated insulin action [15, 18–20]. However, some studies identified IKKβ as the relevant molecular target for insulin sensitization because this kinase could induce serine phosphorylation of insulin receptor substrate (IRS)-1 and attenuate insulin signaling [21, 22].

In this study, we aimed to identify the role of NF-κB in the development of skeletal muscle insulin resistance. We determined whether overactivation of NF-κB played an important role in lipid-induced insulin resistance and whether overactivating NF-κB was sufficient to cause insulin resistance in C2C12 skeletal muscle cells. To achieve these aims, we used small interfering RNA technology to specifically inhibit NF-κB p65 subunit to observe the role of NF-κB in palmitate-induced insulin resistance. And we also used the same technology to inhibit IκBα with the expectation to increase NF-κB activation and observe its effect on insulin resistance.

Result

Concentration dependence of palmitate-induced insulin resistance and NF-κB activation in C2C12 myotubes

We first determined the effects of fatty acids on insulin sensitivity and NF-κB activation in C2C12 myotubes. Palmitate, one of the most common fatty acids found in muscle, was chosen for this study. C2C12 myotubes were incubated for 16 h in the presence of increasing concentrations of palmitate (0, 0.25, 0.50, 0.75, and 1.0 mM) (Fig. 1). Palmitate concentrations of 0.25 mM neither decreased insulin-stimulated 2DG uptake nor activated NF-κB compared with controls. Increasing palmitate concentration to 0.50 or 0.75 mM reduced insulin-stimulated 2DG uptake, activated NF-κB, and decreased IκBα protein levels, and palmitate concentration of 0.75 mM was more effective; there was no additional effect by adding palmitate concentration to 1.0 mM. These data suggest that there exists a good correlation between a fall in insulin-stimulated 2DG uptake and the induction of NF-κB activation and palmitate-induced NF-κB activation involves degradation of IκBα.

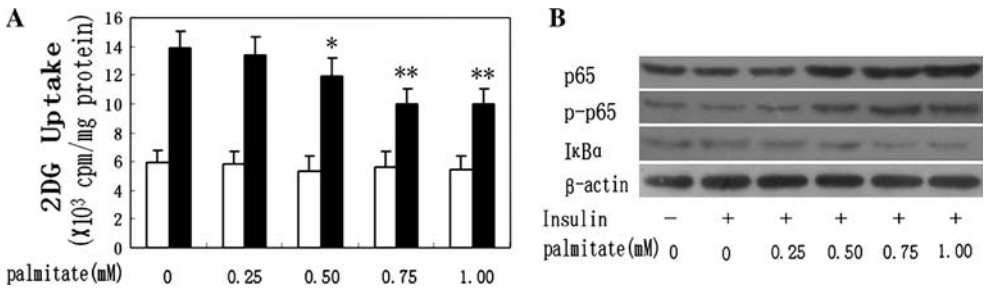

Fig. 1 Concentration dependence of palmitate-induced insulin resistance and NF-κB activation in C2C12 myotubes. C2C12 myoblasts were cultured and differentiated to myotubes. Myotubes were then exposed to various concentrations of palmitate complexed to FFA-free BSA, or to 2% BSA alone for 16 h. **a** [^{3}H]2-deoxy-glucose uptake in the absence (*white bars*) or presence (*black bars*) of 100 nM insulin. Values represent mean ± SD of four independent experiments. * $P < 0.05$; ** $P < 0.01$ versus insulin control. **b** The representative immunoblots of NF-κB protein expression and phosphorylation, IκBα protein expression in cultured C2C12 cells were analyzed by western blot analysis. Similar results were obtained in other two experiments

siRNA-mediated gene knockdown

To test whether siRNA could modulate gene expression in C2C12 myotubes, we, respectively, treated these cells with three NF-κB p65 siRNAs (Rela siRNA 1, 2, 3) and three IκBα siRNAs (Nfkbia siRNA 1, 2, 3). NF-κB p65 and IκBα gene expression was analyzed by RT–PCR and western blotting, carried out in C2C12 myotubes collected at different intervals after a 24-h transfection with 100 nM of each siRNA (Fig. 2). Transfection reagent alone and control siRNA transfection had no significant effect on the expression of target genes. Twenty-four hours after transfection, Rela siRNA1 inhibited p65 mRNA by 80%, Rela siRNA2 inhibited p65 mRNA by 50%, but Rela siRNA3 showed no significant effect on p65 mRNA expression. The percentages of inhibition of IκBα mRNA were 78% (Nfkbia siRNA1), 57% (Nfkbia siRNA2), and 58% (Nfkbia siRNA3). Seventy-two hours after transfection, the effects of siRNAs on target gene expression were still appreciable to a similar extent (Fig. 2b, f). NF-κB p65 and IκBαmRNA downregulation were both paralleled by a reduction in the protein abundance (Fig. 2d, h). Twenty-four hours after transfection, Rela siRNA1 reduced p65 protein abundance by 79% and Nfkbia siRNA1 reduced IκBα protein abundance by 82%. Seventy-two hours after transfection, Rela siRNA1 reduced p65 protein abundance by 72% and Nfkbia siRNA1 reduced IκBα protein abundance by 78%.

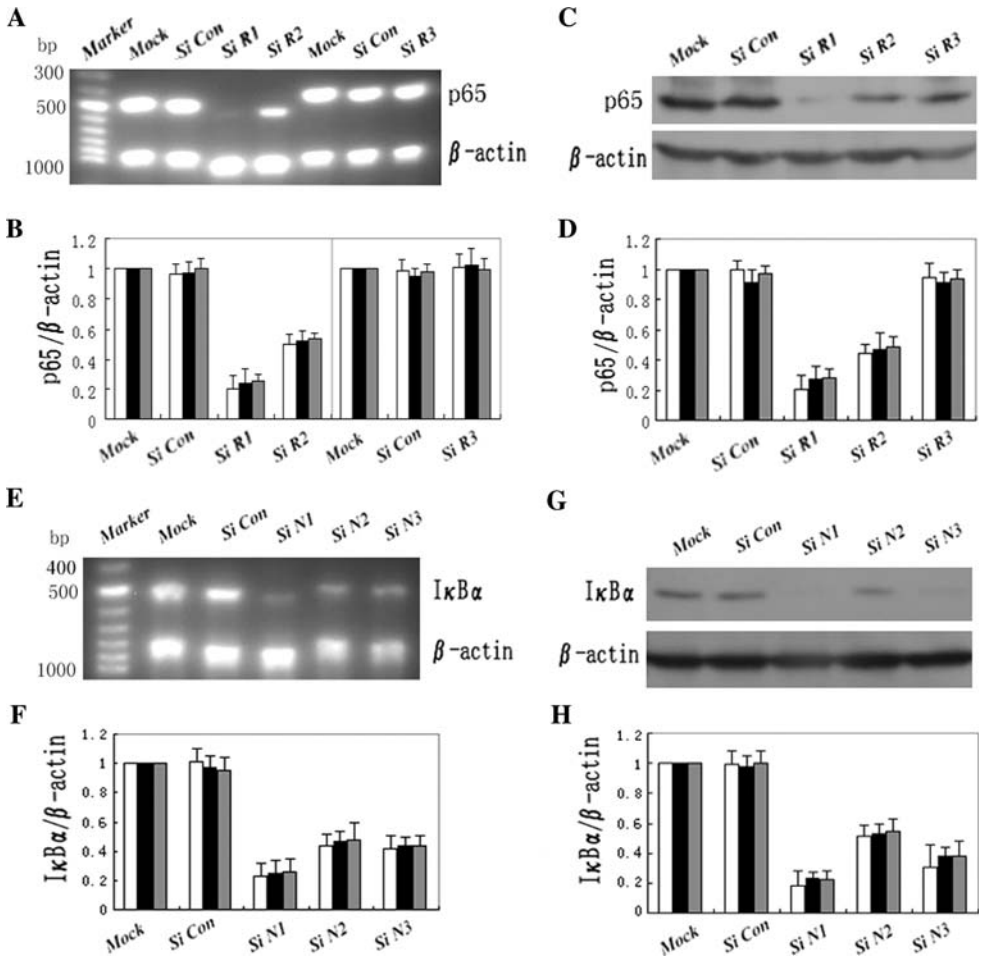

Fig. 2 Target gene expression after the respective treatment with p65 siRNAs or IκBα siRNAs in C2C12 myotubes. **a**–**d** Expression of p65 after treatment with p65 siRNAs. **e**–**h** Expression of IκBα after treatment with IκBα siRNAs. Representative (**a**) RT–PCR and (**c**) western blot experiments illustrated p65 mRNA and protein expression, and representative (**e**) RT–PCR and (**g**) western blot experiments illustrated IκBα mRNA and protein expression at 24 h after transfection. β-actin was used as a loading control. In Fig. 1a, the first four bands of p65 were 417 bp (nos. NM_009045, nucleotides 662–1078) and the last three bands were 351 bp (nos. NM_009045, nucleotides 2330–2680). Quantification of **b** p65 mRNA, **d** p65 protein, **f** IκBα mRNA, and **h** IκBα protein expression levels was shown at 24 h (*white bars*), 48 h (*black bars*), 72 h (*gray bars*) after transfection. *Mock* transfection reagent exposed cells, *Si Con* control siRNA, *Si R* Rela siRNA, *Si N* Nfkbia siRNA. Data show mean ± SD of three independent experiments

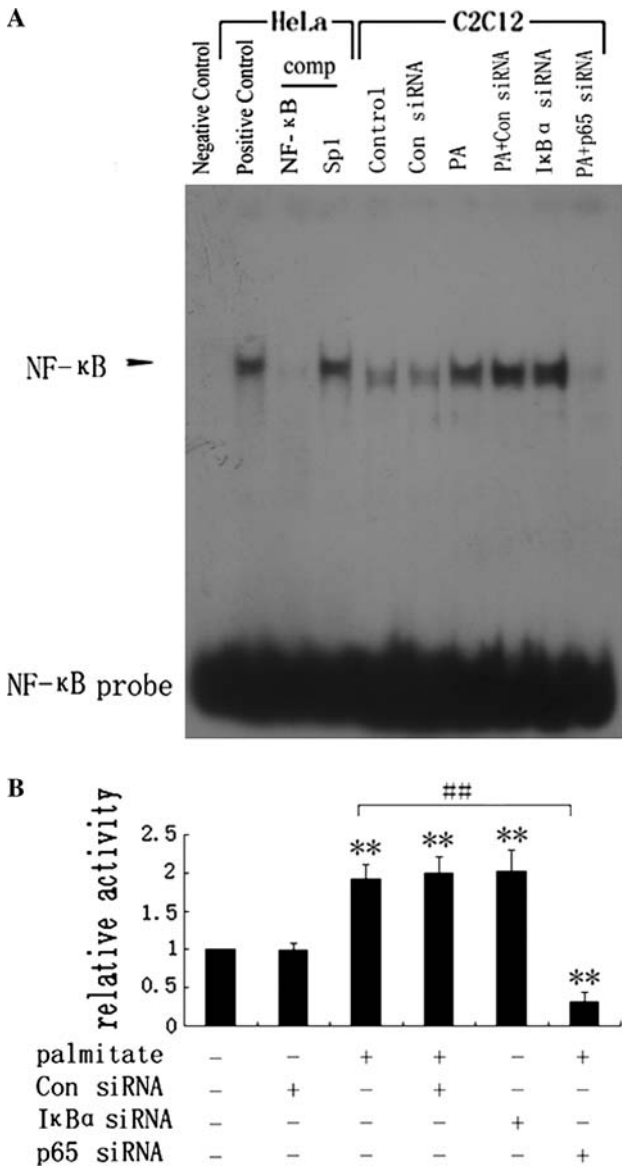

Fig. 3 Effect of palmitate, IκBα siRNA, and co-action of palmitate and p65 siRNA on NF-κB activity. C2C12 myoblasts were differentiated to myotubes in 10-cm dishes. Fully differentiated cells were transfected with transfection regents, control siRNA, IκBα siRNA, or p65 siRNA. Twenty-four hours after transfection, cells were incubated for 16 h in the absence or presence of 0.75 mM palmitate. **a** EMSA using ^{32}P-labeled NF-κB oligonucleotide probe and nuclear extracts from the indicated cell lines. *Lanes 5–10* nuclear extracts of C2C12 myotubes were prepared and analyzed for NF-κB binding as indicated under various conditions. *Lane 1* (negative control), without cell extract; *lane 2* (positive control), HeLa nuclear extract; *lane 3* (cold competition), HeLa nuclear extract, with labeled NF-κB oligonucleotide and unlabeled 100-fold excess of self-oligonucleotide; *lane 4* (non-specific competition), HeLa nuclear extract, with labeled NF-κB oligonucleotide and unlabeled 100-fold excess of irrelevant Sp1 oligonucleotide. *comp* cold competition with double-stranded oligonucleotide, *PA* palmitate, *Con siRNA* control siRNA. **b** Densitometry values for the DNA binding on EMSA in C2C12 myotubes (normalized to the corresponding untreated control). Values presented mean ± SD of three independent experiments. ** $P < 0.01$ versus control. ## $P < 0.01$ versus palmitate pretreated cells as indicated

Rela siRNA1 and Nfkbia siRNA1 were chosen to perform further experiment.

Regulation of NF-κB activation by palmitate, IκBα siRNA, or co-action of palmitate and p65 siRNA

We next compared the effect of palmitate and IκBα siRNA on NF-κB activation and determined the effect of p65 siRNA on palmitate-induced NF-κB activation. A total of 5 μg of protein from each nuclear extract was analyzed for DNA-binding activity by electrophoretic mobility shift assay (EMSA). Negative control experiment was performed without cellular extracts; positive control was performed with HeLa nuclear extract; competition assays with a 100-fold excess of cold NF-κB and cold Sp1 oligonucleotides were performed to establish the specificity of the reaction. No signal was found in the reactions without cellular extracts, and the reaction was proved to be specific because cold NF-κB but not cold Sp1 decreased the DNA-binding activities of NF-κB in HeLa nuclear extract (Fig. 1a). In relation to controls, C2C12 myotubes treated with palmitate and transfected with IκBα siRNA both approximately doubled NF-κB activation. And palmitate-induced NF-κB activation could be largely blocked by pretransfection of myotubes with NF-κB p65 siRNA (Fig. 3a, b).

Regulation of insulin-stimulated 2DG uptake and GLUT4 protein translocation by palmitate, IκBα siRNA, or co-action of palmitate and p65 siRNA

We reasoned that if palmitate-induced insulin resistance was mediated through NF-κB, inhibiting NF-κB expression should prevent defects in insulin action, and other NF-κB activators might lead to analogous effects. To test this hypothesis, we transfected p65 siRNA into C2C12 myotubes to determine the effect of NF-κB on palmitate-induced insulin resistance, and we also transfected IκBα siRNA into myotubes to determine whether increasing NF-κB expression was sufficient to cause insulin resistance (Fig. 4a, b). Palmitate significantly reduced absolute and net insulin-stimulated 2DG uptake. Net insulin-stimulated 2DG uptake was inhibited by as much as 48% after incubation with palmitate for 16 h. Myotubes transfected with NF-κB p65 siRNA for 24 h and followed by treatment with palmitate for 16 h prevented palmitate-mediated decreases in insulin-stimulated 2DG uptake. Exposure of myotubes to IκBα siRNA for 24 h also reduced absolute and net insulin-stimulated 2DG uptake. Net insulin-simulated 2DG uptake was inhibited by 30% compared with control.

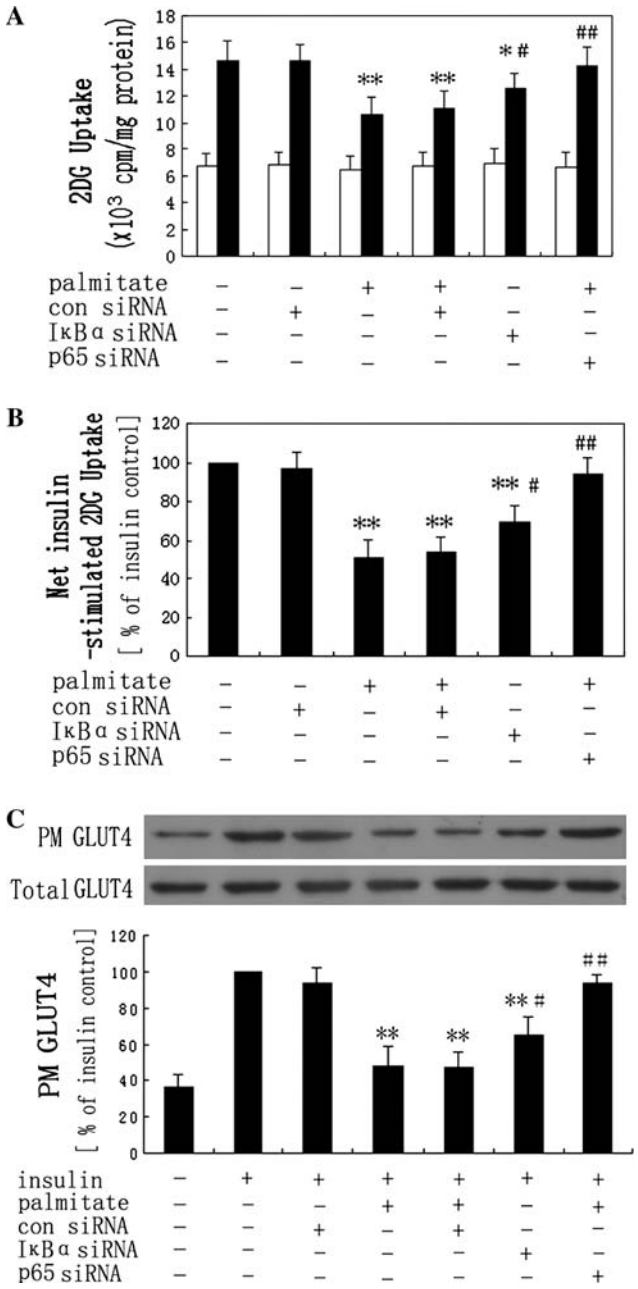

Fig. 4 Measurement of glucose uptake and GLUT4 protein expression in C2C12 myotubes. Fully differentiated cells were first transfected with transfection regents, control siRNA, IκBα siRNA, or p65 siRNA, and then incubated in the absence or presence of 0.75 mM palmitate. **a** [^{3}H]2-deoxy-glucose uptake was measured in the absence (*white bars*) or presence (*black bars*) of 100 nM insulin. Each experiment was repeated four times **b** Net insulin-stimulated 2DG uptake (insulin 2DG uptake − basal 2DG uptake). **c** Immunoblotting was performed to measure PM GLUT4 and total GLUT4 protein content in the absence or presence of insulin. A representative immunoblot and PM GLUT4 densitometry of three independent experiments were shown. Data show mean ± SD. ** $P < 0.01$ versus insulin control. # $P < 0.05$; ## $P < 0.01$ versus palmitate and insulin pretreated cells

Insulin regulates the uptake of glucose into skeletal by redistributing the tissue-specific glucose transporter GLUT4 from intracellular vesicles to the cell surface. We then measured insulin-responsive GLUT4 in C2C12 myotubes. Western blot analysis showed the total GLUT4 contents of skeletal muscle cells have no significant differences in different treatment groups. However, insulin-induced GLUT4 translocation, as assessed by the appearance of GLUT4 in the plasma membrane fraction of skeletal muscle cells, was decreased by 52% in palmitate pretreated cells and by 35% in IκBα siRNA transfected cells. NF-κB p65 siRNA blocked palmitate-mediated decreases in GLUT4 translocation (Fig. 4b). These results matched the regulation of insulin-stimulated glucose transport observed under identical conditions.

Effect of palmitate, IκBα siRNA, or co-action of palmitate and NF-κB siRNA on insulin signaling proteins

To further clarify whether modification of NF-κB expression affect insulin signaling proteins, we next determined insulin-stimulated protein expression and phosphorylation of IRS and Akt. At the present of insulin, palmitate caused a 1.8-fold increase in S632/S635 phosphorylation of IRS-1 and a 54% decrease in S473 phosphorylation of Akt. Addition of p65 siRNA before treatment with palmitate largely prevented these changes. IκBα siRNA transfection caused a 0.7-fold increase in IRS-1 phosphorylation and a 31% decrease in Akt phosphorylation compared with control (Fig. 5a, b).

Discussion

Our data confirm palmitate infusion in C2C12 myotubes decreases insulin-stimulated 2-DG uptake, reduces IκBα levels, and induces NF-κB activation (Fig. 1). This result is consistent with the idea that activation of IKK-β/IκB/NF-κB pathway is related to palmiate-induced skeletal muscle insulin resistance [14, 23]. However, whether NF-κB plays a pivotal role in the development of insulin resistance is disputable. We reasoned that if NF-κB overexpression played a crucial role in palmitate-induced insulin resistance, then inhibiting NF-κB activity could reverse this event, and specifically increasing NF-κB expression in some way independent of palmitate might result in analogous effects; if NF-κB overexpression was only an concomitant phenomenon, specifically overactivating NF-κB would have no effect on insulin action.

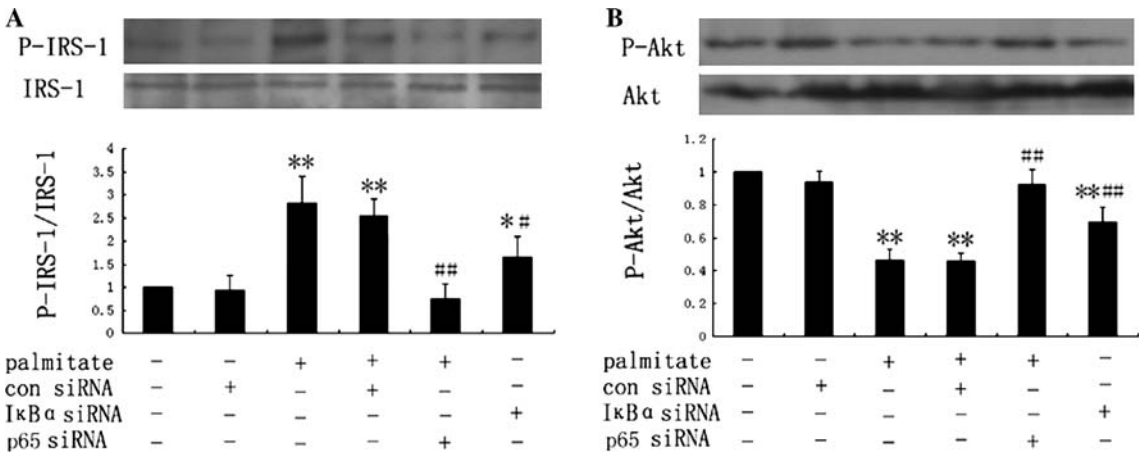

Fig. 5 Measurement of insulin-induced Akt and IRS protein expression and phosphorylation in C2C12 myotubes. C2C12 cells (5×10^6/well) were differentiated to myotubes in a 6-well plate. Fully differentiated cells were first transfected with transfection regents, control siRNA, IκBα siRNA, or p65 siRNA, and incubated in the absence or presence of 0.75 mM palmitate, then incubated for 20 min with 100 nM insulin. The protein levels and phosphorylation of IRS-1 (**a**) and Akt (**b**) were measured by western blot analysis. Data show mean ± SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$ versus control. # $P < 0.05$; ## $P < 0.01$ versus palmitate pretreated cells

To achieve these aims, we successfully down-regulated NF-κB expression by transfection of RelA siRNA into cultured cells (Fig. 2a–d). We also wanted to establish NF-κB overexpression cell model to determine whether over-activating NF-κB was sufficient to cause insulin resistance. IκBα is a specific inhibitor of NF-κB. In basal states, IκBα forms a complex with p50/p65 therefore NF-κB is maintained in an inactive form. After stimulation, IκBα is phosphorylated and degraded, which frees NF-κB to translocate to the nucleus. Furthermore, newly synthesized IκBα could enter the nucleus and bind NF-κB, thereby terminating NF-κB activation. Hence, knocking down IκBα would increase free NF-κB dimerides and enhance NF-κB translocation [24]. We used IκBα siRNA to inhibit IκBα expression and our results showed cells pretreated with palmitate or transfected with IκBα siRNA each doubled NF-κB activation (Fig. 3). This result is consistent with the ideas that degradation of IkB proteins is a fundamental step for NF-κB activation [25] and no other signal-dependent events than those leading to the degradation of IκBα are necessary for nuclear translocation of NF-κB [24].

This investigation confirms that NF-κB activation is required for palmitate-induced insulin resistance and further reveals that NF-κB overexpression is sufficient to impair insulin sensitivity in C2C12 myotubes. This interpretation is based on the following findings: (1) Palmitate augmented NF-κB activation, diminished insulin-stimulated 2-DG uptake and GLUT4 translocation, impaired insulin signaling in C2C12 myotubes. (2) NF-κB p65 siRNA treatment inhibited NF-κB activation and blocked detrimental effects of palmitate on insulin actions. (3) Enhancing NF-κB activation by transfection with an IκBα targeting RNAi also induced decreases in 2-DG uptake and defects in the signaling pathway. These results imply that NF-κB per se plays an important role in the pathogenesis of insulin resistance in skeletal muscle cells and the effect of palmitate is mediated through the activation of NF-κB. And a further implication of our data is that the effects of palmitate on insulin action seem to be a little more potent than that of IκBα siRNA (Figs. 4 and 5), which might imply some NF-κB-independent mechanism activated by palmitate is involved in aggravating the damage but is not sufficient to cause insulin resistance.

Although investigation of the metabolic actions in vitro models might reduce responsiveness to metabolic effects, investigations of in vitro models enable biochemical and molecular studies to be conducted that are substantially more complex to conduct in vivo. The data presented in this study are consistent with a series of in vivo and in vitro studies demonstrating a mechanistic link between activation of pro-inflammatory pathways and insulin resistance. Cai et al. [26] demonstrated that constitutive activation of NF-κB in the liver by transgenic hepatocyte-specific overexpression of IKK-β led to an increased local and systemic insulin resistance in mice that were fed with a normal diet. Expression of IkB-DR (degradation-resistant IkB mutants that acting in blockage of NF-κB activation) in the liver could protect mice from insulin resistance induced by IKK-β expression, or by high fat diet. Hence hepatocyte-specific overexpression IKK-β contributes to insulin resistance probably via overexpression of NF-κB. In vivo studies have demonstrated that inactivation of IKK-β prevents fat-induced insulin resistance in skeletal muscle by blocking fat-induced defects in insulin signaling and action [27]. Then cytological study has revealed that preventing IKK

activation or directly inhibiting NF-κB translocation both prevent decreases in insulin-stimulated 2DG uptake caused by palmitate [15]. Therefore, inactivation of IKK-β leads to inhibition of its downstream protein, NF-κB might be a very important mechanism to protect against insulin resistance. Recent study has found that lipid-induced pPKCε could directly phosphorylate NF-κB and transducing pPKCε protein to the L6 skeletal muscle cells reduced glucose uptake and increased amount of pNF-κ B and NF-κB [18]. Our study demonstrated specifically activation of NF-κB by ablation of IκBα could impair response of skeletal muscle cells to insulin action. It seems to be a reasonable possibility that NF-κB is the most important proinflammatory molecular-inducing insulin resistance.

Our data show that NF-κB overactivation induces insulin resistance through the attenuation of insulin-signaling cascade. Overexpression of NF-κB induced insulin-stimulated phosphorylation (Ser636/639) of IRS, reduced insulin-stimulated phosphorylation (Ser473) of Akt and translocation of GLUT4 (Figs. 5 and 4b). However, for there is no evidence that NF-κB has phosphatase activities, NF-κB might not directly affect insulin-signaling pathway. How NF-κB exerts its action on insulin action is in need for further research. We guess that transactivation of specific gene targets and internal stress state of cells are major mechanisms. Activation of NF-κB participates in the control of transcription of over 150 target genes including inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules. NF-κB has often been termed as 'central switch' of the immune response and stress response [28]. Its target genes TNF-α and interleukin might play a fundamental role in the pathogenesis of skeletal muscle insulin resistance and type 2 diabetes mellitus [29–31]. What is interesting is that exogenous administration of TNF-α or interleukin has also been proved to enhance activation of NF-κB [32–34]. So, when inflammatory pathway is activated, there possibility exists positive feedback loops to activate NF-κB. At the same time, NF-κB also enacts a response by promoting the transcription of genes whose products alleviate the stress condition or even limit NF-κB activation [28]. Taken together, decompensated NF-κB overactivation can lead to cellular stress and an overproduction of wild-type proteins, some of the proteins might impair insulin action. Hence, NF-κB probably acts as 'central switch' not only in cell stress response, but also the development of insulin resistance.

In conclusion, our data demonstrate palmitate-induced insulin resistance in skeletal muscle cells requires the activation of NF-κB. Our study further provided evidence that activation of NF-κB per se is sufficient to decrease response of skeletal muscle cells to insulin action. The results suggest the blockage of NF-κB overexpression seems to be an important strategy to prevent and cure skeletal muscle insulin resistance.

Materials and methods

Cell culture

Mouse C2C12 myoblasts (ATCC, USA) were maintained at 37°C in DMEM containing 10% fetal bovine serum (Hyclone, USA). When cells reached confluence, the medium was switched to the differentiation medium containing DMEM and 2% horse serum (Hyclone, USA), which was changed every other day. After four additional days, the differentiated C2C12 cells had fused into myotubes.

FFA treatment

Palmitate-containing media were prepared by conjugation of palmitate (Sigma, USA) with FFA-free BSA (Sigma, USA), by a method modified from that described by Pickersgill et al. [35]. Stock palmitate solutions (9 mM), complexed to 24% (w/v) BSA, were prepared. This gave a final molar ratio of palmitate/BSA of 2.5. Palmitate were dissolved in 3 ml ethanol, and diluted in DMEM. An optically clear solution was obtained upon heating to 60°C with continuous agitation and an equal volume of ice-cold 48% FFA-free BSA was immediately added. Samples were stored at −20°C until required. Fatty acid stocks were diluted to appropriate concentrations in serum-free DMEM. Cells were washed thrice with phosphate-buffered saline (PBS) prior to the addition of fatty acid-containing medium. Control cells were incubated in serum-free DMEM containing 2% FFA-free BSA.

siRNA preparation and transfection

Three small interfering RNAs (Rela siRNA 1, 2, 3) were designed targeting murine NF-κB p65 and three siRNAs (Nfkbia siRNA 1, 2, 3) were designed targeting murine IκBα. The sequences were taken from GenBank accession nos. NM_009045 (nucleotides 740–760, 990–1010, and 2632–2652) and NM_010907 (nucleotides 591–611, 911–931, and 784–804). These siRNAs and negative control siRNAs were synthesized by GenePharma (Shanghai, China). Selected siRNA target sequences were also analyzed by BLAST research to insure that they did not have significant sequence homology with other murine genes. The negative control siRNAs have been tested and they show no effects on any mRNA. When cells were differentiated, myotubes were transfected with 100 nM siRNA complexes or only with transfection regents (mock

transfection) using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to manufacturer's instructions. After 24 h of transfection, cells were washed with DMEM. Transfected cells were then used for further experiments.

RNA extraction and RT–PCR analysis

Total RNA was isolated from 6-well plates using the TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. After extraction, reverse transcription was performed immediately on 100 ng of RNA using the RT–PCR kit (Fermentas, Lithuania) according to manufacturer's instructions. Two pairs of primers were designed for Rela. A 417-bp product (nucleotides 662–1078) was generated using the primers 5′-CAGACCGCAGTATCCA TAG-3′ and 5′-GCACATCAGCTTGAGAAAA-3′ to test the gene knockdown effect of Rela siRNA1 and 2; and a 351-bp product (nucleotides 2330–2680) was generated using the primers 5′-TATCAAGTGTCTTCCTCCACG-3′ and 5′-GCTAGAAAGAGCAAGAGTCCA-3′ to test the gene knockdown effect of Rela siRNA3. Primers for Nfkbia were 5′-ACCCCTCTACATCTTGCC-3′ and 5′-TT TTGCCACTTTCCACTTA-3′ (526 bp products, nucleotides 662–1078). Primers for β-actin were 5′-TGAGAGGG AAATCGTGCGAGAC-3′ and 5′-TGAGGGACTTCCTG TAACCACT-3′ (861 bp product). PCR was performed using Taq DNA polymerase kit (Promega, USA) using the following conditions: initial denaturation at 94°C for 4 min followed by 30 cycles at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, then 72°C for 10 min. PCR products were separated on 2% agarose gel. For the semi-quantification, an image of gel was captured, and the intensity of the bands was quantitated using Bio-Rad Quantity One software (Bio-Rad, Canada). The relative expression level was normalized to that of β-actin.

Total protein extraction

C2C12 myotubes were suspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris–HCl, pH 7.5, at 4°C, Shenergy Biocolor, China) supplemented with protease inhibitor (Shenergy Biocolor) and incubated on ice for 30 min. Lysates were cleared by centrifugation ($10000\times g$, 30 min), and the protein concentrations determined by using the Bradford method.

Plasma membrane fraction fractionation of C2C12 cells

Cells were plated onto 10-cm-diameter dishes. After treatment, cells were washed thrice on ice with PBS. Cell culture dishes were scraped into ice-cold HES buffer (255 mM sucrose, 4 mM disodium EDTA, 20 mM HEPES, pH 7.4) containing protease inhibitors and homogenized by PRO200 Homogenizer (pro Scientific Inc., USA). The homogenate was centrifuged at $19000\times g$ for 20 min. The pellet from this spin was resuspended in HES buffer, layered onto a 1.12 M sucrose cushion in 20 mM HEPES, and 1 mM disodium EDTA, and centrifuged at $100000\times g$ for 1 h. The interphase of the sucrose cushion was collected and pelleted at $40000\times g$ for 20 min. This PM-containing pellet was resuspended in PBS plus protease inhibitors. All centrifugations were performed at 4°C with a Beckman Ultraspeed centrifuge (Optima L-80XP, Germany).

Immunoblotting

Proteins (20 μg) were separated by SDS–PAGE on separation gels and transferred to polyvinylidene difluoride membrane (Roche, German). Western blot analysis was performed using antibodies against NF-κB p65, phospho-p65 (serine 276), IκBα, GLUT4, IRS-1, phospho-IRS-1 (Ser636/639), Akt, phospho-Akt (Ser473), β-actin (CST Inc., USA), followed by peroxidase-conjugated anti-rabbit IgG secondary antibody (CST Inc.). Detection was achieved using the EZ-ECL chemiluminescence detection kit (CST Inc.). Size of detected proteins was estimated using protein molecular mass standards (CST Inc.). Quantification of band intensity was performed using Bio-Rad Quantity One software. Linearity of staining on western blots was confirmed by loading increasing amounts of cell lysate and scanning to confirm relative increases of specific band intensity.

Nuclear protein extracts, EMSAs

DNA-binding proteins were extracted from myotubes by the method of Andrews and Faller [36], which utilizes hypotonic lysis followed by high salt extraction of nuclei. Cells were plated onto 10-cm-diameter dishes. After experimental treatment, cells were scraped into 1.5 ml of cold PBS, pelleted for 10 s and resuspended in 400 μl of cold Buffer A [10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF, 5 μg/ml aprotinin, and 2 μg/ml leupeptin, at 4°C] by flicking the tube. Cells were allowed to swell on ice for 15 min and then lysed with 20 μl of Nonidet P-40 (10%) and centrifuged at $16000\times g$ at 4°C for 1 min. The pellet from this spin was washed twice in buffer A and resuspended in 50 μl of cold Buffer C [20 mm HEPES–KOH (pH 7.9) at 4°C, 25% glycerol, 400 mm NaCl, 50 mm KCl, 1.5 mm $MgCl_2$, 0.2 mm EDTA, 0.5 mm DTT, and the protease inhibitor cocktail] and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 10 min at 4°C at $16000\times g$

and the supernatant fraction (containing DNA binding proteins) was stored at −80°C. Nuclear extract concentration was determined by using the Bradford method.

EMSA was performed using double-stranded oligonucleotides (Promega) for the consensus binding site of the NF-κB nucleotide (5′-AGTTGAGGGGACTTTCCCAGG C-3′). Consensus binding site of the Sp1 nucleotide was used as non-specific competition (5′-ATTCGATCGGGGC GGGGCGAGC-3′). Ligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 1 μl of 10× kinase buffer, 1 μl of T4 polynucleotide kinase (10 U/μl), and 2.2 μl of [γ-^{32}P] ATP (5000 Ci/mmol at 10 mCi/ml, Furui Biotech Corp., China) incubated at 37°C for 10 min. The reaction was stopped by adding 90 μl of TE buffer [10 mm Tris–HCl, pH7.4, and 1 mm EDTA]. Crude nuclear proteins were incubated for 10 min on ice in 5× binding buffer. Labeled probe was added and the reaction was incubated for 15 min at room temperature. Non-radiolabeled oligonucleotides were at 100-fold excess to the ^{32}P-labeled probes for specific and non-specific competition for DNA binding. Competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. Protein–DNA complexes ran on a non-denaturing 5% acrylamide gel in TBE. The gel was dried and exposed to X-ray film. Quantification of band intensity was performed using Bio-Rad Quantity One software.

[^{3}H]2-deoxy-glucose uptake

C2C12 myoblasts (5×10^5/well) were differentiated to myotubes in a 24-well plate. After experimental treatments, the myotubes were rinsed thrice in KRP-Hepes buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1.2 KH_2PO_4 and 1 mM $CaCl_2$). The myotubes were then stimulated with or without insulin (100 nM) for 20 min. At the end of stimulation period, the myotubes were incubated for 10 min in KRP-Hepes buffer containing [^{3}H]2-deoxyglucose (0.5 μCi/ml, Perkin Elmer, USA). Glucose transport was stopped by washing thrice with ice-cold PBS in the present of 10 μM cytochalasin B (Sigma). Cells were collected in 0.1 N NaOH. The incorporated radioactivity into the cells was measured by liquid scintillation counting. The level of non-specific radioactivity in the cell pellet was determined by adding 10 μM cytochalasin B to a set of control samples before incubation with [^{3}H]2-deoxyglucose [37]. These background counts were subtracted from all the values. The values of 2DG uptake (cpm) in each experiment were adjusted by the protein content measured by the Bradford method. In each experiment, glucose uptake was derived from the mean of duplicate determinations.

Statistical analyses

Significant differences were established by Student's *t*-test or one-way analysis of variance (ANOVA) with SNK-q test for multiple comparisons among the groups, using the statistical software SPSS 16.0 (SPSS Inc., USA). Results are expressed as means ± SD of at least three independent experiments. Differences were considered significant if $P < 0.05$.

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