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Pomy Barma, Sushmita Bhattacharya, Anirban Bhattacharya, Rakesh Kundu, Suman Dasgupta, et al.. Lipid induced overexpression of NF- κ B in skeletal muscle cells is linked to insulin resistance. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1792 (3), pp.190. 10.1016/j.bbadis.2008.11.014 . hal-00562887

HAL Id: hal-00562887

<https://hal.science/hal-00562887>

Submitted on 4 Feb 2011

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Accepted Manuscript

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PII: S0925-4439(08)00247-0
DOI: doi:[10.1016/j.bbadis.2008.11.014](https://doi.org/10.1016/j.bbadis.2008.11.014)
Reference: BBADIS 62899

To appear in: *BBA - Molecular Basis of Disease*

Received date: 20 July 2008
Revised date: 9 November 2008
Accepted date: 21 November 2008



Please cite this article as: Pomy Barma, Sushmita Bhattacharya, Anirban Bhattacharya, Rakesh Kundu, Suman Dasgupta, Anindita Biswas, Shelley Bhattacharya, Sib Sankar Roy, Samir Bhattacharya, Lipid induced overexpression of NF- κ B in skeletal muscle cells is linked to insulin resistance, *BBA - Molecular Basis of Disease* (2008), doi:[10.1016/j.bbadis.2008.11.014](https://doi.org/10.1016/j.bbadis.2008.11.014)

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Lipid induced overexpression of NF- κ B in skeletal muscle cells is linked to insulin resistance

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Abstract:

Lipid induced NF- κ B activation is known to be associated with insulin resistance and type2 diabetes. Here we show that incubation of L6 skeletal muscle cells with palmitate significantly increased NF- κ B p65 and NF- κ B p50 expression along with their phosphorylation. NF- κ B p65 siRNA inhibited palmitate induced overexpression of NF- κ B p65 indicating palmitate effect on transcriptional activation. RT-PCR and Real time PCR experiments also showed a significant increase in NF- κ B p65 gene expression due to palmitate. Overexpression of NF- κ B p65 by palmitate was linked to impairment of insulin activity. Palmitate effect on NF- κ B gene and protein expression was found to be mediated by phospho-PKC ϵ as calphostin C (an inhibitor of PKC) and ϵ V1 (PKC ϵ translocation inhibitor) significantly reduced NF- κ B expression. To understand the underlying mechanism, we purified NF- κ B and pPKC ϵ from palmitate incubated skeletal muscle cells and their interaction in cell free system demonstrated the transfer of phosphate from PKC ϵ to NF- κ B. This prompted us to transduce pPKC ϵ to the skeletal muscle cells. These cells showed increased amount of pNF- κ B and NF- κ B. Excess of NF- κ B p65 pool thus created in the cells made them insulin resistant. Addition of NF- κ B p65 siRNA and SN50 inhibited palmitate induced NF- κ B p65 expression indicating NF- κ B regulation of its gene expression. Increase of NF- κ B did not affect the activation of IKK/I κ B indicating NF- κ B p65 expression to be a distinct effect of palmitate. Since NF- κ B p65 is linked to several diseases, including type2 diabetes, this report may be important in understanding the pathogenicity of these diseases.

Key words: Insulin resistance; NF- κ B; Free fatty acid; PKC ϵ ; Insulin signaling.

1. Introduction

NF- κ B plays a critical role in implementing the pathogenesis in a number of inflammatory diseases including type2 diabetes [1-3]. It is well known that free fatty acids (FFAs) are linked to insulin resistance and type 2 diabetes [4-9]. Increase in tissue lipid levels is inversely related to insulin sensitivity [10, 11] and short term FFA infusion induces insulin resistance [12-17]. Patients suffering from insulin resistance and type2 diabetes show excess plasma FFA level and lowering of the FFA level normalized insulin sensitivity in these patients [18-21]. Interestingly, inhibitor of κ B kinase (IKK) has been implicated in the mediation of FFA induced insulin resistance [17, 21]. Inhibitory κ B proteins (I κ Bs) restrict the NF- κ B dimer in the cytoplasm, phosphorylation of I κ B α by IKK induces its polyubiquitination and proteasomal degradation thus making NF- κ B (p50/p65) free for its translocation to the nucleus [3, 22, 23]. NF- κ B, a family of transcription factors, regulates the expression of proinflammatory and anti-apoptotic genes [24, 25]. FFAs promote NF- κ B activity that in turn targets gene products involved in the pathogenesis of insulin resistance [21, 26].

FFA has been shown to activate NF- κ B and its nuclear translocation that compromised insulin sensitivity in skeletal muscle cells; inhibitors of IKK/I κ B/ NF- κ B pathway prevent FFA induced impairment of insulin activity [22]. A link between NF- κ B and type2 diabetes is probably maintained by inflammatory cytokines. Levels of a number of pro-inflammatory cytokines are elevated in type2 diabetes along with the activation of NF- κ B [3, 27]. Incubation of skeletal muscle cells with palmitate increased IL-6 gene expression and secretion via activation of NF- κ B and PKC which causes

insulin signaling defects [28]. FFA may induce insulin resistance via the involvement of activated NF- κ B and novel PKCs [28-30]. We have recently demonstrated that PKC ϵ plays a major role in FFA induced insulin resistance in skeletal muscle cells and adipocytes by downregulating insulin receptor (IR) gene expression [31-33]. However, whether the activation of PKC ϵ by FFA could be linked to NF- κ B was not addressed by us.

In the present investigation we have witnessed an unexpected event. In our attempt to illustrate whether NF- κ B is associated with the FFA induced phospho-PKC ϵ effect on IR gene downregulation, we detected an increase in NF- κ B protein in the skeletal muscle cells incubated with palmitate. Earlier reports in this field demonstrated that FFA impaired insulin activity in skeletal muscle cells by activating NF- κ B through IKK/I κ B pathway. We have found that FFA i.e., palmitate increased the expression of NF- κ B protein by triggering NF- κ B gene. This being an unusual finding is expected to attract attention of other investigators since NF- κ B is related to the expression of a spectrum of genes that are associated with various kinds of pathogenic conditions including insulin resistance and type2 diabetes.

2. Materials and Methods

2.1. Materials

All tissue culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, USA. Palmitate and porcine insulin were purchased from Sigma Chemical Company, St. Louis, MO, USA. [3 H] 2-deoxyglucose (Specific activity: 12.0

Ci/mmol) and γ -³²P ATP (Specific activity: 6000 ci/mmol) were from GE Healthcare, Asia Pacific Region, Singapore. Calphostin C, PKC ϵ translocation inhibitor peptide EAVSLKPT (ϵ V1) and SN50 were purchased from Calbiochem, Darmstadt, Germany. Antibodies utilized included anti-NF- κ B p65, anti-pNF- κ B p65, anti-NF- κ B p50, anti-pNF- κ B p50, anti-IKK β , anti-pIKK, anti-I κ B α , anti-pI κ B α , anti-PKC ϵ and anti-pPKC ϵ antibodies were purchased from Santa Cruz Biotechnology Inc., California, USA. Alkaline phosphatase conjugated anti-rabbit, anti-goat and anti-mouse secondary antibodies and Control and NF- κ B p65 (h) siRNA were also purchased from Santa Cruz Biotechnology Inc., California, USA. GFP-Glut 4 construct was a kind gift from Dr. Jeffrey E. Pessin, Chairman and Professor of the Department of Pharmacology, The University of Illinois, USA. The other chemicals and reagents used in the present investigation were purchased from Sigma Chemical Co., St. Louis MO, USA.

2.2. Cell culture and treatments

L6 skeletal muscle cell line was procured from the National Centre for Cell Science, Pune, India and was cultured in a similar manner as described by us previously [32, 33]. Briefly, L6 skeletal muscle cells were cultivated in DMEM medium supplemented with 10% fetal calf serum, 25 mM glucose, penicillin (100 U/ml) and streptomycin (100 μ g/ml) in a humidified 95% O₂/5% CO₂ atmosphere at 37°C. Confluent cells were trypsinized and reseeded in DMEM containing glucose, reduced serum (2%) and antibiotics. Media was changed every 2 days and finally myotube differentiation was completed after 6 days of culture. Myotubes were treated with palmitate (lipid-containing media were prepared by conjugation of free fatty acid with bovine serum albumin by

following our earlier description [32]) at different doses (0.5, 0.75 and 1.0 mM) and time periods (0 h, 2 h, 4 h and 6 h) without or with insulin (100 nM) or without any of them (control). For experiments with different inhibitors such as SN50 (50 µg/ml), Calphostin C (100 nM) and PKCε translocation inhibitor εV1 (150 µg/ml) treatments are described at respective places.

2x10⁵ cells were plated in a six well culture plate and NF-κB p65 siRNA (20 pmol/µl) was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEM reduced serum medium according to the manufacturer's protocol. After 24 h of transfection, cells were washed with DMEM. Transfected cells were then used for different experiments.

On termination of incubations, cells were washed twice with ice-cold PBS and harvested with trypsin (0.25%)–EDTA (0.5 mM). Cell pellets were resuspended in lysis buffer (1% NP-40, 20 mM HEPES (pH 7.4), 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 mM PMSF) and sonicated on ice for 10 min (with 20 s pulse, 2 min between each pulse). Lysates were centrifuged for 10 min at 10,000 g and protein concentrations of supernatant were determined by the method of Lowry et al. [34].

2.3. [³H] 2-deoxyglucose uptake

[³H] 2-deoxyglucose uptake in L6 myocytes was conducted according to an earlier method described from our laboratory [35]. L6 myocytes were serum starved overnight in Kreb's Ringer Phosphate (KRP) buffer (12.5 mM HEPES, pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.6 mM

Na₂HPO₄) supplemented with 0.2% bovine serum albumin. Cells were incubated separately for 4h in the presence or absence of palmitate (0.75 mM) followed by 30 min incubation with porcine insulin (100 nM). Incubations in the absence of any of these chemicals were taken as the control. After 25 min, [³H] 2-deoxyglucose (0.4 nmoles/ml) was added to each of the incubations 5 min before the termination of experiment. Cells were washed thrice with ice-cold KRP buffer in the presence of 0.3 mM phloretin to correct the glucose uptake data from simple diffusion and non-specific trapping of radioactivity. Cells were harvested with trypsin (0.25%) -EDTA (0.5 mM), solubilized with 1% NP-40 and [³H] 2-deoxyglucose (2-DOG) was measured in a Liquid Scintillation counter (Perkin Elmer, Tri-Carb 2800TR).

2.4. Gel Filtration and Immunoaffinity Chromatography

To prepare NF- κ B rich fraction, gel filtration chromatography was performed on a Sephadex G-75 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column (2x50 cm) equilibrated with Tris-Cl buffer (10 mM Tris-Cl, pH-7.4) by following an earlier description from this laboratory [36]. The flow rate was maintained at 1 ml/min and 1.0 ml fractions were collected just after loading the skeletal muscle cell lysate. A standard gel filtration molecular weight marker from Sigma Chemical Co., St. Louis, USA, was run to determine the approximate molecular weight of the eluted proteins. Fractions around 66 kDa were pooled, lyophilized to reduce the volume and subjected to 10% SDS-PAGE. To identify NF- κ B p65 there, the gel was transferred to PVDF membrane for Western blot analysis. It was further purified by immunoaffinity chromatography where CNBr activated Sepharose 4B beads were conjugated with the

anti-NF- κ B p65 antibody. Coupling of the antibody was carried out by following the procedure previously described from this laboratory. Briefly, NF- κ B p65 antibody coupled Sepharose 4B beads were loaded on a 5 ml column. Unbound antibody was removed by washing with coupling buffer (pH-8.5) followed by acetate buffer (0.1 M, pH-4.0). The remaining active groups on the beads were blocked with glycine buffer (0.2 M, pH-8.0). The sample was loaded on the column and kept overnight at 4°C. The unbound proteins were eluted with 10 mM Tris-HCl buffer, pH-7.4 while NF- κ B p65 bound to the ligand was eluted with elution buffer (10 mM Tris-HCl, pH -7.4) containing 2.0 M KI as a chaotropic agent. The volume of each eluted fraction was 1.0 ml. The fractions were collected until the O.D. at 280 nm reached the baseline, pooled and dialyzed against the buffer (10 mM Tris-HCl, pH -7.4) and lyophilized. The purity of NF- κ B p65 was examined in a 10% SDS-PAGE where it gave a single protein band which also crossreacted with anti-NF- κ B p65 antibody.

2.5. Electrophoresis and immunoblotting

60 μ g protein from the lysates of control and treated cells were resolved on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA 01730) through transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol, pH 8.5) for 1.5 h. Western blot analysis was performed by following the method described previously from this laboratory [31-33, 35] using phospho-specific antibodies such as anti-pPKC ϵ (anti-rabbit), anti-pNF- κ Bp65 (anti-rabbit), anti-pI κ B α (anti-mouse), anti-pIKK (anti-goat), anti-pNF- κ B p50 (anti-rabbit) antibodies. A concurrently run gel was immunoblotted with non-phospho antibodies such as anti-NF- κ B p65 (anti-rabbit), anti-

I κ B α (anti-rabbit), anti-IKK β (anti-goat), anti-NF- κ B p50 (anti-rabbit), anti-PKC ϵ (anti-rabbit) and anti- β Actin antibodies. Membrane bound primary antibodies were visualized using corresponding secondary antibodies at 1:1000 dilutions, which were tagged with alkaline phosphatase and were developed with corresponding substrates, 5-bromo 4-chloro 3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT). Band intensities were quantified by utilizing Image J software (NIH, Bethesda, MD).

2.6. Reverse transcription PCR and Real-time quantitative PCR

RNA isolation from control and treated skeletal muscle cell incubations was performed using the Tri reagent RNA extraction kit. RT-PCR of isolated RNA was carried out using RT kit from First strand cDNA synthesis kit, Fermentas Life Sciences, Revert AidTM, Hanover, MD, USA, following a previous description from this laboratory [33]. Alteration in NF- κ B p65 gene expression was observed in semi-quantitative RT-PCR and it was thereafter confirmed with the help of real-time quantitative PCR (qPCR) by using iCycler (Bio-Rad) real-time PCR machine and Dynamo SYBR Green real-time qPCR kit (Finzymes, Espoo, Finland). 3 μ g of total RNA from each sample was reverse transcribed and PCR was performed with gene specific primers in a total volume of 20 μ l in the following real-time PCR conditions: initial activation step (95°C -15 min), cycling step (denaturation 95°C - 30 s, annealing at 55°C - 30 s, and finally extension for 72°C -30 s x 40 cycles) followed by their melt curve analysis (55–60°C, 15 s, 40x). A house keeping gene, *gapdh*, was simultaneously amplified in separate reactions and acted as an internal control. The CT value was corrected by CT reading of corresponding *gapdh*

controls. Data from six determinations (means \pm SEM) are expressed as relative expression level. The following primer pairs were used in real time PCR:

NF- κ B sense: 5' -CCATCAGGGCAGATCTCAAACC-3';

antisense: 5' -GCTGCTGAAACTCTGAGTTGTC-3', product of 380 bp;

gapdh: sense: 5'- GCCATCAACGACCCCTTC-3';

antisense: 5'-AGCCCCAGCCTTCTCCA- 3' product of 290 bp.

The cycle number at which NF- κ B p65 (RelA) transcripts were detectable (CT) was normalized to that of *gapdh*, referred to as Δ CT.

2.7. EMSA and Supershift assay

Nuclear extracts were prepared from control, palmitate, NF- κ B p65 siRNA and SN50 treated cells as described previously [37]. The specific oligonucleotide probes were end labelled with γ^{32} P-dATP using T4 polynucleotide kinase (NEB, Beverly, MA). The probes used in EMSA were designed in analogy with known proximal promoter sequence of NF- κ B gene. The probes were incubated with 10 μ g of nuclear extracts in a 20 μ l of binding reaction (20% glycerol, 100 mM Tris-HCl pH-8.0, 300 mM KCl, 25 mM MgCl₂, 500 μ g/ml BSA) on ice for 45 min. For supershift assays, wild type probe was incubated with 10 μ g nuclear extract in a 20 μ l of binding reaction for 20 min on ice and then 4 μ g of NF- κ B p65 specific antibody was added, followed by incubation on ice for an additional 30 min. Reaction mixture were resolved on 5% polyacrylamide gel using 0.5xTBE running buffer at 150 V for 3.5h in a Bio-Rad electrophoresis system. The gels

were transferred to 3M Whatman filter paper, dried and exposed to films. The probes used in EMSA are mentioned.

Wild type probe: 5'-AGTTGAGGGGACTTTCCCAGGC-3';

Mutant probe: 5'-AGTTGAGGGCGACTTTACCAGGC-3'

2.8. *GFP-Glut 4 Transfection, transduction of pPKC ϵ protein and immunofluorescence*

GFP-Glut 4 transfection was carried out according to our previous description [35]. Briefly, L6 skeletal muscle cells were plated on 60 mm plate containing cover slips and maintained in an air/CO₂ (19:1) atmosphere in DMEM supplemented with 10% (v/v) FBS and 100 μ g/ml penicillin /streptomycin. After 8h, cells were washed with DMEM free from FBS. Plasmid DNA of GFP-Glut 4 (2 μ g) was used to transfect 2×10^5 cells on each 60 mm plate with Lipofectamine reagent in accordance with the manufacturer's protocol (Invitrogen, USA). After 48h of transfection (transfection efficiency: 65-70%) cells were incubated without (control) or with insulin (100 nM) for 6h. To a batch of GFP-Glut 4 transfected cells pPKC ϵ was delivered as pPKC ϵ -Triton X-100 complex and then subjected to insulin incubation. On termination of the incubation, cells on the cover slips were fixed in paraformaldehyde (3.5%) and mounted on glass slides. The cover slips were examined for translocation of GFP-Glut 4 under laser scanning confocal microscope (Leica Corp., Rockleigh, NJ).

Delivery of pPKC ϵ protein into the skeletal muscle cells was performed by following a method described earlier [38] with certain modifications. Purified pPKC ϵ was obtained in the similar manner as described below under "Autoradiography of

radiolabelled PKC ϵ and NF- κ B” except the use of non-radioactive ATP in the incubation medium. Briefly, Triton X-100 was sonicated for 1 min and mixed with 10 μ g of purified pPKC ϵ protein in PBS (Phosphate buffer saline, 50 mM, pH-7.4 and 0.15 mM NaCl) to form a complex at room temperature for 30 min. This complex mixture was then kept at 4°C for another 30 min. The complex formed was diluted 10 times with DMEM containing 10% FBS. Cells grown to 95% confluency were incubated with the protein-Triton X-100 complex for 2h and then washed extensively with DMEM. A portion of skeletal muscle cells thus transfected with pPKC ϵ protein was processed for immunofluorescence study in the following manner. Skeletal muscle cells were seeded on sterile uncoated 22 mm coverslips and grown for 2 days in DMEM with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95%O₂ / 5%CO₂ at 37°C. Cells were then subjected to fixation with 3.5% paraformaldehyde in HANKS’ salt solution containing 20 mM HEPES (pH 7.0), permeabilized in phosphate buffered saline (PBS) containing 0.1% Triton X-100 and 1% BSA for 5 min followed by incubation with anti-pPKC ϵ antibody. After treatment with fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:100), coverslips were mounted in glycerol/PBS and viewed under fluorescence microscope. (Olympus BX51 microscope, Tokyo, Japan). The images were captured with cool snap pro camera. When immunofluorescence study demonstrated transfection of pPKC ϵ into the cells, the rest of the cells transfected with pPKC ϵ were incubated with insulin to observe pPKC ϵ effect on insulin activity.

2.9.a. Autoradiography of radiolabelled PKC ϵ and NF- κ B

Cell lysates from palmitate incubated cells containing [γ - 32 P] ATP was centrifuged for 10 min at 10,000 g and supernatant was subjected to Microcon filter devices (Millipore Co., Bedford, MA, USA) through YM-50 and then YM-100, filtrate from YM-100 was collected and solubilized with PBS buffer (50 mM, pH-7.4). This was subjected to immunoprecipitation by following a previous description from our laboratory [35]. Briefly, 200 μ g protein from cell lysates was incubated overnight at 4°C with 2 μ g pPKC ϵ antibody. 50 μ l of protein A-Sepharose was added to each tube and incubated at 4°C for 2 h followed by centrifugation at 10,000 g. The immuno-complex of pPKC ϵ formed was dissolved in 500 μ l of 0.1% CHAPS in PBS. Purity of 32 pPKC ϵ was examined in 10% SDS-PAGE prior to autoradiography where it gave a clear single band around 90 kDa indicating the homogeneity of 32 pPKC ϵ . 10 μ g of 32 pPKC ϵ was then added to a reaction mixture containing 10 μ M MgCl $_2$, 10 μ g purified NF- κ B p65. The reaction was carried out for 20 min at 25°C and terminated by addition of 40 μ l of 1x SDS sample buffer [39]. Autoradiography was performed for both 32 pPKC ϵ (after its purification) and the reaction mixture where it was incubated with NF- κ B. 20 μ l of aliquot in each case was resolved on 10 % SDS-PAGE, transferred to PVDF membrane and autoradiographed on Kodax BiomaxMR film (Eastman Kodak Co., Rochester, NY).

2.9.b. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) where the F value indicated significance, means were compared by a post hoc multiple range test.

Densitometric analysis was done by using Image J software. All values were means \pm SEM.

3. Results

3.1 Incubation of skeletal muscle cells with palmitate augmented phosphorylation and expression of NF- κ B p50 and NF- κ B p65

We previously demonstrated that free fatty acid i.e. palmitate, effects insulin resistance in skeletal muscle cells by inhibiting insulin receptor gene expression and that in turn abrogated insulin signaling pathway [32, 33]. NF- κ B is known to be related to insulin resistance and type2 diabetes. We wanted to examine the role of NF- κ B in fatty acid induced impairment of insulin signaling pathway. Fig. 1A shows that palmitate significantly increased the phosphorylation of NF- κ B p65 and NF- κ B p50 as compared to control ($p < 0.01$ for p65 and $p < 0.05$ for p50) while insulin had no effect. We used anti-NF- κ B antibody as loading control to substantiate the increase in NF- κ B phosphorylation due to fatty acid. Surprisingly, we observed that palmitate markedly increased NF- κ B p50 and NF- κ B p65 protein expression ($p < 0.01$) as compared to control or insulin incubation of skeletal muscle cells. In a time kinetics study, it was observed that incubation of skeletal muscle cells with palmitate augmented both phosphorylation and protein expression of NF- κ B p65 at 4 and 6h which corresponded to the decrease of insulin stimulated 3 [H] 2-DOG uptake by palmitate (Fig. 1 B). A significant increase of NF- κ B p65 protein and phosphorylation at the same time by palmitate indicates an

involvement of NF- κ B in the impairment of insulin activity. Fig. 2A shows that palmitate increased NF- κ B p50 and NF- κ B p65 at a dose of 0.5 and 0.75 mM, whereas 0.25 mM had no effect; there was no additional increase by 1.0 mM over the 0.75 mM. Transfection of NF- κ B p65 siRNA into the skeletal muscle cells followed by palmitate incubation did not show increase in NF- κ B protein expression indicating palmitate effect on pNF- κ B p65 transcription.

3.2. Purification of palmitate-induced NF- κ B p65 protein

Skeletal muscle cells incubated without (control) or with palmitate were lysed by sonication, centrifuged at 10,000 x g for 10 min and supernatant was loaded on 10% SDS-PAGE gel. Fig. 3A demonstrates that palmitate incubation overexpressed p65 protein. The supernatant was then loaded on Sephadex G-75 column and the elution profile showed an increase of 65 kDa protein due to palmitate incubation. The eluted fractions of this area were pooled, lyophilized and subjected to Western blot analysis with anti-NF- κ B p65 antibody. The inset of Fig. 3A demonstrates that both A1 (control) and A2 (palmitate treated) fractions crossreacted with anti-NF- κ B p65 antibody; there was a substantial increase of NF- κ B p65 in palmitate incubated fractions. A2 fraction was then loaded on an immunoaffinity column where anti-NF- κ B p65 antibody was linked to Sepharose 4B (Fig. 3C). Fractions eluted with buffer (P1) or eluted with KI as chaotropic agent (P2) were lyophilized and examined by Western blot analysis. P2 was found to be pure NF- κ B p65 protein (inset of Fig. 3C). Purity of p65 was verified in SDS-PAGE which showed a clear single band protein at 65 kDa zone (data not shown).

3.3. Palmitate overexpressed p65 NF- κ B gene

Palmitate induced increase in NF- κ B protein expression and its inhibition by NF- κ B p65 siRNA suggests palmitate effect on NF- κ B gene expression. To examine this, skeletal muscle cells were incubated in varied time periods with palmitate and RT-PCR showed that NF- κ B p65 gene expression was detectably increased at 2h; it was further increased at 4h and expression level remained same at 6h (Fig. 4A). In NF- κ B p65 siRNA transfected L6 muscle cells, palmitate incubation did not increase NF- κ B gene expression (Fig. 4B). Real time PCR data demonstrated a highly significant ($p < 0.001$) quantitative increase of NF- κ B p65 mRNA due to palmitate (Fig. 4C).

3.4. Inhibition of palmitate induced NF- κ B expression reduced its activation

The pathway of NF- κ B activation is well known. Activation of inhibitor of κ B kinase (IKK) phosphorylates I κ B, an inhibitor of NF- κ B; phosphorylated I κ B is degraded releasing NF- κ B for its translocation to the nucleus which regulates expression of a number of genes. Fatty acid induced elevation of NF- κ B activity has been reported earlier [21, 26]. To examine whether fatty acid induced overexpression of NF- κ B is related to its activity, we performed EMSA with palmitate incubated skeletal muscle cells. NF- κ B p65 binding activity increased in nuclear extract from palmitate treated cells. Addition of antibody against NF- κ B p65 supershifted the binding complex suggesting that this band is mainly constituted by this subunit, this was not observed in the nuclear extract from control cells. To check the specificity of NF- κ B p65 and DNA interactions, we used a mutant of NF- κ B which did not show any binding activity.

Incubation of NF- κ B p65 siRNA transfected L6 skeletal muscle cells with palmitate significantly inhibited palmitate induced augmentation of NF- κ B p65 binding to DNA. Addition of SN50, a translocation inhibitor of NF- κ B, to palmitate incubation which significantly reduced palmitate induced increase in NF- κ B binding to DNA (Fig. 5A). Two aspects are indicated from these observations - i) increase of NF- κ B expression due to fatty acid is linked to a greater binding to DNA implicating its augmented activation and ii) decrease of NF- κ B binding by NF- κ B p65 siRNA and SN50 suggests regulation of NF- κ B expression by NF- κ B. Palmitate induced phosphorylation of IKK and I κ B was not adversely affected by NF- κ B inhibition indicating reduction of NF- κ B synthesis is not linked to IKK and I κ B activation or deactivation under present experimental conditions (Fig. 5B). Association of NF- κ B in insulin resistance was indicated by the improvement of the inhibition of insulin stimulated [3 H] 2-DOG uptake due to SN50 (Fig. 5C).

3.5. Phosphorylation of PKC ϵ by palmitate is associated with NF- κ B p65 overexpression

We have reported earlier that incubation of skeletal muscle cells with palmitate phosphorylated PKC ϵ [31] and a link between FFA induced NF- κ B activation and novel PKCs has also been demonstrated [40]. These reports prompted us to examine whether overexpression of NF- κ B p65 by palmitate is mediated through PKC ϵ . Fig. 6A shows a time dependent increase in PKC ϵ phosphorylation due to palmitate which increased from 2-4h, incubation at 6h did not show significant difference from 4h. This coincided with the overexpression of NF- κ B p65. Addition of PKC inhibitor, Calphostin C, decreased

palmitate induced overexpression of NF- κ B p65. Incubation of PKC ϵ translocation inhibitor, ϵ V1, with palmitate significantly ($p < 0.001$) reducing palmitate stimulation of NF- κ B p65 expression (Fig. 6B) indicative of involvement of PKC ϵ in the palmitate induced overexpression of NF- κ B p65.

It was intriguing to observe that in control cells non-phospho PKC ϵ was prevalent as compared to its phospho form; while in palmitate incubated cells situation was reverse, there was more phospho form of PKC ϵ than its non phospho form. Increased pPKC ϵ coincided with the increased phospho NF- κ B and higher expression of NF- κ B (Fig. 7A). Use of PKC inhibitor, Calphostin C and NF- κ B translocation inhibitor, SN50 reduced pPKC ϵ and pNF- κ B along with the reduction of NF- κ B expression in palmitate incubated skeletal muscle cells. Inhibition of NF- κ B transcription by NF- κ B p65 siRNA did not alter the status of PKC ϵ or pPKC ϵ indicating PKC ϵ in the upstream of NF- κ B (Fig. 7B). These results implicate palmitate induced pPKC ϵ 's involvement in the activation of NF- κ B which may lead to overexpression of NF- κ B. To examine this, we incubated L6 skeletal muscle cells without (control) or with palmitate in the presence of γ^{32} pATP. Since palmitate induces a constitutive phosphorylation of PKC ϵ [32, 33], radiolabelled phosphate is expected to be incorporated into the PKC ϵ . Palmitate incubated cells were lysed, centrifuged and supernatant was subjected to immunoprecipitation using anti-pPKC ϵ antibody. Purified radiolabelled PKC ϵ was loaded on SDS-PAGE followed by autoradiography which showed that a considerable amount of 32 pPKC ϵ could be obtained from palmitate incubated cells (Fig. 7C). 32 pPKC ϵ was then incubated with NF- κ B p65 in cell free system with the expectation that if activated PKC has kinase activity with NF-

κ B, transfer of radiolabelled phosphate from PKC ϵ to the NF- κ B will occur. On termination of incubation, reaction mixture was subjected to SDS-PAGE followed by autoradiography. From a parallel incubation, after autoradiography radioactive zone on PVDF membrane was cut to determine the radioactive count in a liquid scintillation counter. Fig. 7D demonstrates that radioactive phosphate from 32 pPKC ϵ was transferred to NF- κ B. These results suggest that pPKC ϵ phosphorylates NF- κ B p65 which is translocated to the nuclear region and expresses the NF- κ B p65 gene.

3.6. Transduction of pPKC ϵ to L6 skeletal muscle cells inhibited insulin activity

To examine the possibility that pPKC ϵ may activate NF- κ B p65 which in turn augmented its gene expression, we transduced purified pPKC ϵ into the cells (Fig. 8A). As expected, Western blot analysis showed a significantly greater concentration of pPKC ϵ in the transduced cells in comparison to the control cells. Increase in pPKC ϵ coincided with increased pNF- κ B p65 and overexpression of NF- κ B p65 (Fig. 8B). These results indicate that increase of pPKC ϵ form due to palmitate mediates NF- κ B p65 expression via the activation of NF- κ B p65. If this is true, then pPKC ϵ transduced cells would demonstrate insulin resistance. It can be seen from Fig. 8C and D that insulin stimulated translocation of GFP-Glut4 into the cell membrane and uptake of 3 [H] 2-DOG, which are dependable markers to assess insulin activity, were significantly abrogated in pPKC ϵ transduced cells. Presumably, cells loaded with pPKC ϵ would have increased expression of NF- κ B and that in turn attenuated insulin activity.

3.7. Acute and chronic effects of palmitate on NF- κ B expression

To compare the acute and chronic effects of palmitate on NF- κ B expression, we have used two different doses i.e. 0.75 mM and 0.25 mM. Throughout our experiments we observed the acute effect of palmitate using the concentration of 0.75 mM by following previous descriptions [32, 33, 41-43]. To observe the chronic effect, L6 skeletal muscle cells were incubated for longer durations i.e. from 16 h to 48 h. With 0.75 mM palmitate, both NF- κ B p65 gene and protein expressions were at peak at 4 h (as depicted in Fig. 4 & 6) as no additional NF- κ B expression occurred in spite of longer duration of incubation. In contrast, 0.25 mM palmitate showed a chronic effect demonstrating no effect until 8 h. At 16 h p65 gene and protein expressions began to appear, reaching the peak at 24 h since 48 h had no additional effect (Fig. 9A & 9B). Similar results were obtained with pPKC ϵ and pNF- κ B p65 (data not shown) again suggesting 0.75 mM to be an acute dose while 0.25 mM of palmitate produced chronic effect.

4. Discussion

There are two important information generated from the present investigation – (1) overexpression of NF- κ B due to palmitate is associated with insulin resistance and (2) this effect of palmitate is mediated through the activation of PKC ϵ . It is fairly well known that lipid-induced insulin resistance and type2 diabetes is linked to the activation of NF- κ B [1, 2, 5, 22]. Our observation of NF- κ B overexpression due to FFA appeared

serendipitously when we wanted to find a link between FFA induced downregulation of insulin receptor (IR) gene expression, as reported previously from this laboratory [32], and FFA induced activation of NF- κ B. In searching the underlying mechanism about how FFA i.e. palmitate inhibits IR gene and protein expression in skeletal muscle cells, we have found that palmitate phosphorylated PKC ϵ in cytosol which is then translocated to the nuclear region and is associated with the inhibition of IR gene expression [33]. Relationship between novel PKC and NF- κ B has been indicated by several authors [29, 30, 39, 44]. We therefore thought it relevant to investigate whether NF- κ B is associated in this pathway of FFA induced impairment of insulin activity. With this assumption we incubated L6 skeletal muscle cells with palmitate and found a significant increase in NF- κ B activation as reported by others [21, 28], but at the same time we were surprised to observe a remarkable increase of NF- κ B protein in the immunoblot probed with anti-NF- κ B p65 and anti-NF- κ B p50 antibodies.

Another interesting finding is the relationship between NF- κ B p65 protein expression and its phosphorylation. Palmitate at a dose of 0.75 mM induced NF- κ B p65 gene and protein expression could be detected at 2h while an increase in its phosphorylation occurs at not less than 4h incubation. This is the time when phosphorylated NF- κ B p65 crosses the nuclear membrane and binds to DNA of skeletal muscle cells in response to palmitate. These data suggest FFA induced activation of NF- κ B may be associated with its overexpression. However, such time-kinetics events are not similar in chronic incubation with 0.25 mM palmitate. Proximal element of pro-inflammatory response is initiated by IKK which phosphorylates I κ B that inhibits nuclear translocation of NF- κ B. Phosphorylation of I κ B results its ubiquitination and

degradation thus permitting the release of NF- κ B for nuclear translocation to activate gene expression [45]. A short term lipid infusion in human being induces insulin resistance in skeletal muscle and also reduces I κ B levels indicating a relationship between the activation of NF- κ B and insulin resistance [14]. This has been further supported by a report with L6 skeletal muscle cells where palmitate augments nuclear translocation of NF- κ B causing insulin resistance. Inhibition of NF- κ B nuclear translocation due to palmitate also blocks palmitate induced insulin resistance [21]. Thus activation of NF- κ B by palmitate is associated with the impairment of insulin activity. We have observed another facet of the palmitate- NF- κ B relationship. Palmitate induced overexpression of NF- κ B in skeletal muscle cells is also related to insulin resistance. Incubation of NF- κ B p65 siRNA transfected cells with palmitate reduced its inhibitory effect on insulin stimulated [³H] 2-DOG uptake. Since this inhibition is very specific, it implicates that overexpression of NF- κ B due to palmitate could be associated with its activation. This is further strengthened by the findings that demonstrate a reduction of NF- κ B binding to DNA due to NF- κ B p65 siRNA and SN50, as these inhibitors have not affected palmitate induced IKK and I κ B phosphorylation. These results indicate that increase in internal cellular pool of NF- κ B having an influence on its enhanced binding to DNA, may be via the increased turnover of IKK/I κ B/NF- κ B pathway. This event is also reflected by the impairment of insulin stimulated Glut4 translocation and glucose uptake due to pPKC ϵ transduction which increased both activation and expression of NF- κ B.

While searching for the possible mechanism of palmitate induced NF- κ B gene and protein expressions, we investigated the involvement of a novel PKC i.e. PKC ϵ for

the following reasons. A report from this laboratory showed that palmitate induced phosphorylation of PKC ϵ in the cytosol of skeletal muscle cells was independent of PDK1. pPKC ϵ is then translocated to the nuclear region and on associating with the inhibition of insulin receptor gene expression compromises with the insulin sensitivity resulting insulin resistance [32, 33]. A recent report demonstrated that PKC ϵ plays an important role in mediating lipid induced hepatic insulin resistance, as knocking down of PKC ϵ expression reverses fat induced impairment of hepatic insulin signaling [46]. PKC ϵ has been implicated in regulating NF- κ B activation in thymocytes [44] and cardiomyocytes [29]. When we incubated skeletal muscle cells with palmitate, a detectable increase of PKC ϵ phosphorylation was found to occur at 2h which further increased at 4h remaining at the level till 6h. Pattern of PKC ϵ activation due to palmitate coincided with the expression of NF- κ B which was significantly augmented at 4h. However, this is not an evidence of a direct link of activated PKC ϵ with NF- κ B expression. We therefore used Calphostin C, a general PKC inhibitor, which effectively decreased NF- κ B expression. The convincing evidence came from PKC ϵ translocation inhibitor ϵ V1 experiment. ϵ V1 inhibits the translocation of PKC ϵ and its addition to skeletal muscle cells incubation with palmitate reversed palmitate stimulation of NF- κ B expression. Since ϵ V1 is a specific inhibitor of PKC ϵ translocation, involvement of PKC ϵ in mediating FFA induced NF- κ B expression seems to be a reasonable possibility.

Although these evidences implicate PKC ϵ involvement in NF- κ B expression but they do not answer how PKC ϵ could regulate such an expression. To answer this question, we performed a few experiments to demonstrate a molecular link between

activated PKC ϵ and NF- κ B activation. Phosphorylation and dephosphorylation of NF- κ B is a major regulatory event for NF- κ B nuclear translocation following its dissociation from I κ B α . Although a number of kinases, such as PKA [47-49], casein kinase II [50-52] and PKCs [14, 53] are shown to cause NF- κ B phosphorylation, a clear understanding about different phosphorylatory sites and their role in NF- κ B function remains an important requirement. That the activation of PKC ϵ at the upstream is a need to activate NF- κ B has been suggested by several authors [54-56]. It has also been shown that PKC ϵ is an important signal transducer for RhoA mediated activation of NF- κ B in human monocytes [57]. We have demonstrated that palmitate causes a prevalence of phospho-PKC ϵ form in skeletal muscle cells; isolation and purification of this form followed by incubation with NF- κ B p65 in a cell-free system shows a transfer of phosphate from PKC ϵ to NF- κ B. Hence, pPKC ϵ activates NF- κ B which in turn expresses NF- κ B gene on its translocation to the nucleus. Use of inhibitors also exhibited pPKC ϵ to be an upstream regulator of NF- κ B activation. Interestingly, our findings indicate the existence of an autoregulatory pathway of NF- κ B. This concept of autoregulatory loop has already been put forwarded by some authors by demonstrating that NF- κ B and I κ B α are creating such a loop [58] and serine phosphorylation of I κ B by TNF- α downregulates NF- κ B DNA binding and decreased expression of p65/p50 [59]. We show here that palmitate induced overexpression of NF- κ B p65 gene could be blocked by NF- κ B translocation inhibitor. In summary, our present observation focused certain interesting aspects of lipid induced NF- κ B in skeletal muscle cells. Palmitate augmented NF- κ B gene and protein expression in skeletal muscle cells by inducing the phosphorylation of

PKC ϵ which in turn activates NF- κ B. This increase of NF- κ B in the cellular pool adversely affected insulin sensitivity. Since NF- κ B is a proinflammatory master switch inducing insulin resistance, the present information would be important to devise a therapeutic approach for type2 diabetes.

Acknowledgements:

Authors are grateful to the Head, Department of Zoology, Visva-Bharati University and the Director, Indian Institute of Chemical Biology for extending the facilities required for the present investigation. We appreciate the gift of GFP-Glut 4 construct provided by Dr. Jeffrey E. Pessin, Chairman and Professor of the Department of Pharmacology, The University of Illinois, USA. We also gratefully acknowledge the financial assistance from the Department of Science & Technology (Grant No. VI-D&P/137/06-07/TDT), Ministry of Science & Technology, New Delhi. P. Barma expresses her gratitude to Council of Scientific & Industrial Research, New Delhi; R. Kundu and S. Dasgupta are thankful to University Grants Commission, New Delhi for the grant of research fellowship.

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Figure Legends

Fig. 1. Palmitate stimulation of both NF- κ B p65 and NF- κ B p50 protein expression and phosphorylation is associated with insulin resistance. A) L6 skeletal muscle cells were incubated with 0.75 mM palmitate for 4h and changes in the NF- κ B p65 and NF- κ B p50 protein expression and level of phosphorylation were detected by Western blot analysis by using specific antibodies raised against NF- κ B p65, phospho- NF- κ B p65, NF- κ B p50 and phospho-NF- κ B p50. β actin was used as loading control. B) L6 skeletal muscle cells were incubated with 0.75 mM palmitate at different time periods (from 0 to 6h), cells were lysed and subjected to Western blot analysis. To estimate the [3 H] 2-deoxyglucose uptake for each treatment, skeletal muscle cells treated with insulin (I) or palmitate (P) or none (C) were sonicated, centrifuged and counted in a liquid scintillation counter according to the procedure described in the text. Densitometric analysis was done by

using Image J software. Means \pm SEM was determined from five independent experiments; * $p < 0.01$ as compared with the insulin; # $p < 0.05$ in comparison to insulin.

Fig. 2. Effect of varied doses of palmitate on NF- κ B expression. A) L6 skeletal muscle cells were incubated with different doses of palmitate for 4h, on termination of incubation cell lysates were subjected to Western blot analysis by using specific antibodies raised against NF- κ B p65 and NF- κ B p50. β actin was used as loading control. B) NF- κ B p65 siRNA blocks the palmitate induced expression of NF- κ B p65. Skeletal muscle cells were incubated with palmitate (P) or palmitate plus NF- κ B p 65 siRNA (P+siRNA) or any of them (C). Western blot analysis was performed using anti- NF- κ B p65 antibody. Densitometric analysis was done by using Image J software. Means \pm SEM was calculated from five independent experiments; * $p < 0.01$; ** $p < 0.001$ in compared to control.

Fig. 3. Purification of NF- κ B protein. A) Control and palmitate incubated (4h) L6 skeletal muscle cell lysates were resolved on a 10% SDS-PAGE. A clear band at the 65 kDa region indicates overexpression of NF- κ B p65 protein. B) L6 skeletal muscle cells were incubated with palmitate and cell lysates after centrifugation was loaded on a Sephadex G-75 column. A control was run parallelly to compare the effect of palmitate. Separately collected fractions (such as A1, A2 and A3) from control and palmitate treated cell lysates were immunoblotted with anti-NF- κ B p65 antibody. The lanes A1 and A2 shows the immunoreactive bands of NF- κ B (inset of Fig. 3B) . C) The pooled fractions from gel filtration chromatography were lyophilized and loaded on a immunoaffinity

column where CNBr activated Sepharose 4B was coupled with anti-NF- κ B antibody . P1 (unbound protein) and P2 (bound fraction eluted with 2M KI) peaks were collected, subjected to Western blot analysis, P2 protein immunoreacted with anti-NF- κ B antibody (inset of Fig. 3C).

Fig. 4. Palmitate induced NF- κ B gene expression. A) Total RNA was isolated from L6 skeletal muscle cells incubated with palmitate for different time periods (0-6h) and RT-PCR was performed using specific primers for NF- κ B. A house keeping gene *gapdh* was amplified simultaneously to serve as an internal control. B) L6 skeletal muscle cells incubated without (C) or with palmitate (P) or palmitate plus NF- κ B p65 siRNA were subjected to RT-PCR analysis. C) L6 skeletal muscle cells incubated with or without palmitate for 4h were subjected to Real-time PCR experiment in which NF- κ B mRNA expression was quantitatively measured using *gapdh* as an internal control. Values represent means \pm SEM of three independent experiments. * $p < 0.001$ in comparison to control.

Fig. 5. Relationship between NF- κ B overexpression and activation. A) Nuclear extracts (NE) were prepared from L6 skeletal muscle cells incubated for 4h with palmitate, palmitate plus NF- κ B p65 siRNA or SN50 or siRNA control or without any of them (control) followed by EMSA. Both wild type (lanes 2-7) and mutant (lanes 8-11) DNA sequences were used to determine the specificity of NF- κ B binding. The lanes are: lane1- probe, lane2- control, lane3- palmitate, lane4- palmitate+NF- κ B p65 siRNA, lane5- palmitate+SN50, lane6- palmitate+siRNA control and lane7- palmitate+anti-NF- κ B

antibody (represents the supershift assay) and lanes 8 to 11- denotes the mutant probe to verify the specificity of NF- κ B p65 binding to DNA. B) Levels of palmitate induced phosphorylation of IKK and I κ B were determined in skeletal muscle cells incubated in the presence of palmitate (P) or palmitate plus NF- κ B p65 siRNA (P+NF- κ B siRNA) or palmitate plus SN50 (P+SN50) or without any of them (C) using anti-pIKK and anti-pI κ B α antibodies as probes. Immunoblots for IKK and I κ B α protein served as loading controls. C) L6 skeletal muscle cells incubated with insulin (I), insulin + palmitate (P+I), insulin + palmitate + SN50 (P+I+SN50) or without any of them (control) for 4h to determine [3 H] 2-deoxyglucose uptake. Values represent means \pm SEM of three independent experiments, * p <0.01 (I vs P+I) and ** p <0.05 (P+I vs P+I+SN50).

Fig. 6. Relationship between activated PKC ϵ and NF- κ B p65 protein expression. A) L6 skeletal muscle cells were incubated with palmitate for different time periods (0-6h) and Western blot analysis was performed with anti-pPKC ϵ and anti-NF- κ B p65 antibodies. B) Effect of PKC inhibitor, Calphostin C and PKC ϵ translocation inhibitor ϵ V1 on palmitate induced NF- κ B p65 protein expression. Densitometric analysis was carried out using Image J software. Means \pm SEM was calculated from five independent experiments. * p <0.001 (0 h vs 4 h); ** p <0.001 (P vs Cal C or ϵ V1).

Fig. 7. Palmitate induced phosphorylation of NF- κ B is mediated by pPKC ϵ . (A) L6 myotubes were incubated for 4 h without (control) or with palmitate. On termination of incubation, cells were lysed and immunoblotted with anti-PKC ϵ , anti-pPKC ϵ , anti-pNF- κ B p65 and anti-NF- κ B p65 antibodies. (B) L6 myotubes were incubated for 4 h without

or with palmitate and with different inhibitors as mentioned in the lower panel. SN50 and calphostin C were added to respective incubations 1 h before addition of palmitate. siRNA transfection was described in the method section. After termination of treatment the cells were harvested for Western blot analysis with anti-PKC ϵ , anti-pPKC ϵ , pNF- κ B p65 and anti-NF- κ B p65 antibodies. (C) L6 myotubes were incubated for 4 h without (C) or with palmitate (P) in presence of [γ - 32 P] ATP. Cells were lysed, immunoprecipitated with pPKC ϵ followed by 10 % SDS-PAGE and 32 P-labeled pPKC ϵ was visualized by autoradiography (upper panel). % incorporation of radiolabeled phosphate into PKC ϵ was determined by liquid scintillation counter. (D) For *in vitro* kinase assay, radiolabeled pPKC ϵ was incubated *in vitro* with or without (control) pure NF- κ B p65 protein for 20 min at 25°C. The reaction was terminated by boiling the reaction mixture in SDS sample buffer and resolved in 10% SDS-PAGE followed by autoradiography. % incorporation of radiolabeled phosphate into NF- κ B was determined by liquid scintillation counter.

Fig. 8. Delivery of pPKC ϵ protein into the cells causes impairment of insulin activity. A) pPKC ϵ protein was delivered in L6 skeletal muscle cells and its incorporation was detected by immunofluorescence. B) Control and pPKC ϵ transduced cells (PTC) were subjected to Western blot analysis with anti-pPKC ϵ , anti-pNF- κ B p65 and anti-NF- κ B p65 antibodies. C) GFP-Glut 4 construct was transfected into the skeletal muscle cells and incubated with insulin (I) or I+pPKC ϵ protein or with none (C-control). On termination of incubation, GFP-Glut 4 localization was detected by using laser scanning confocal microscope. D) The same set of incubations was used to determine the [3 H] 2-

deoxyglucose uptake. Values represent means \pm SEM of three independent experiments, * $p < 0.01$ (I vs I + PTC).

Fig. 9. Effect of chronic treatment of L6 myotubes with palmitate on NF- κ B expression. A) L6 myotubes were incubated with palmitate (0.25 mM and 0.75 mM doses) for 16 h, 24 h and 48 h, total RNA was isolated and subjected to RT-PCR analysis. A house keeping gene *gapdh* was amplified simultaneously to serve as an internal control. (B) L6 myotubes were incubated with palmitate (0.25 mM and 0.75 mM doses) for 0-48 h as mentioned in the upper panel and subjected to Western blot analysis with anti-NF- κ B p65 antibody. β actin was used as loading control.

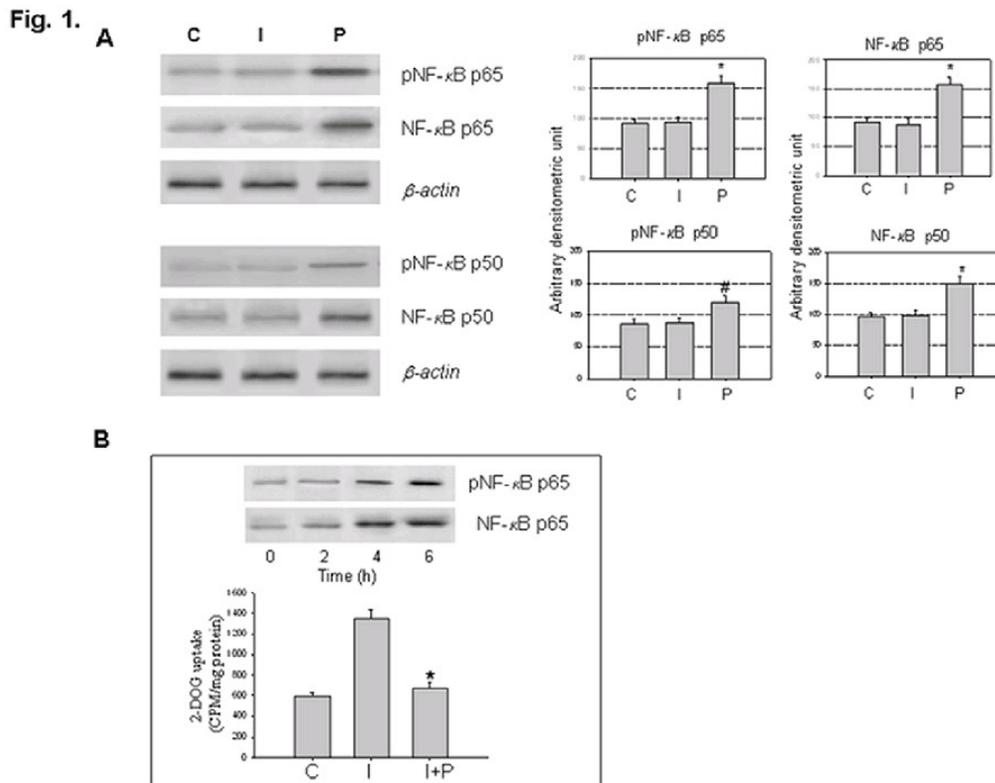
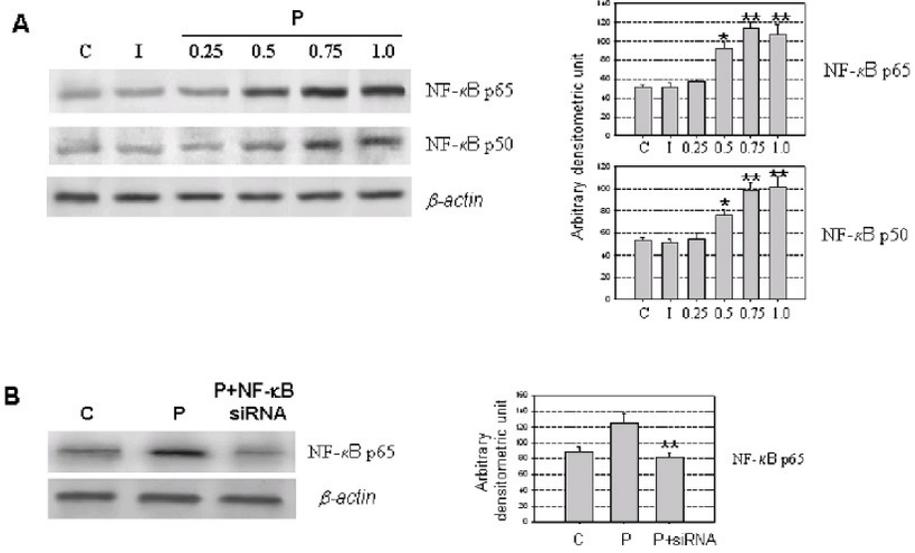


Fig. 2.



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Fig. 3.

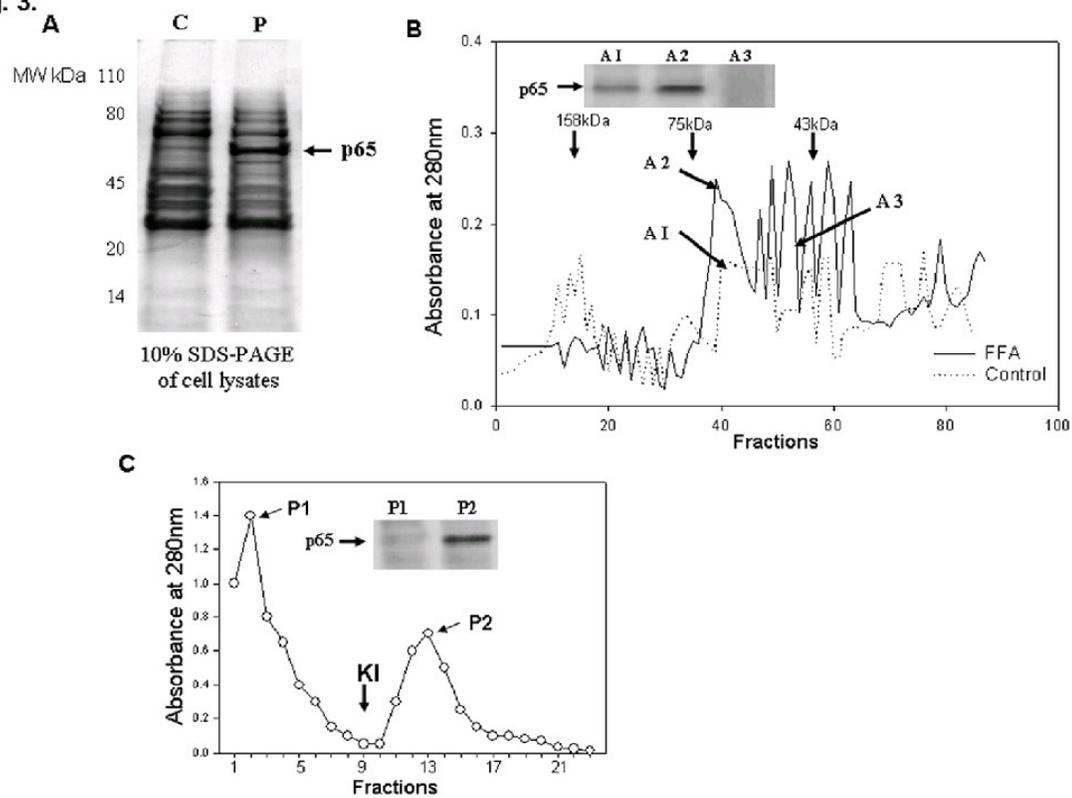
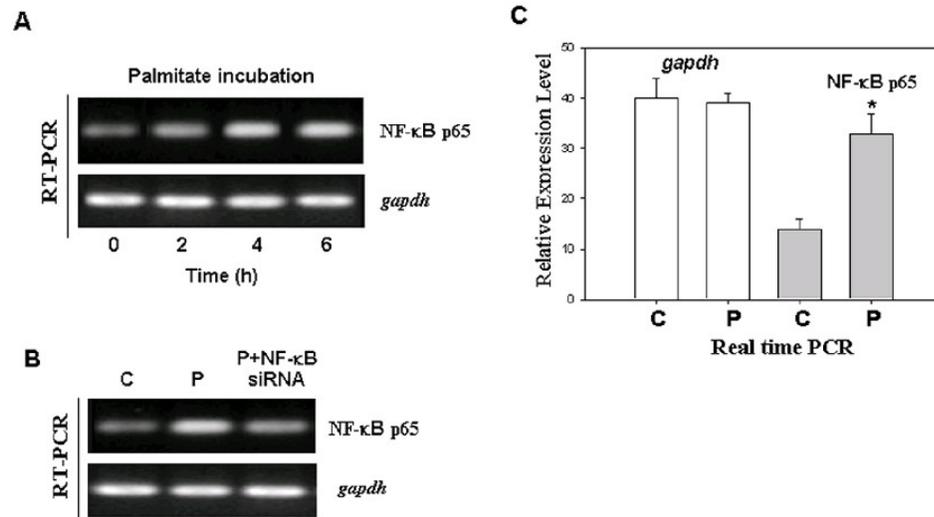
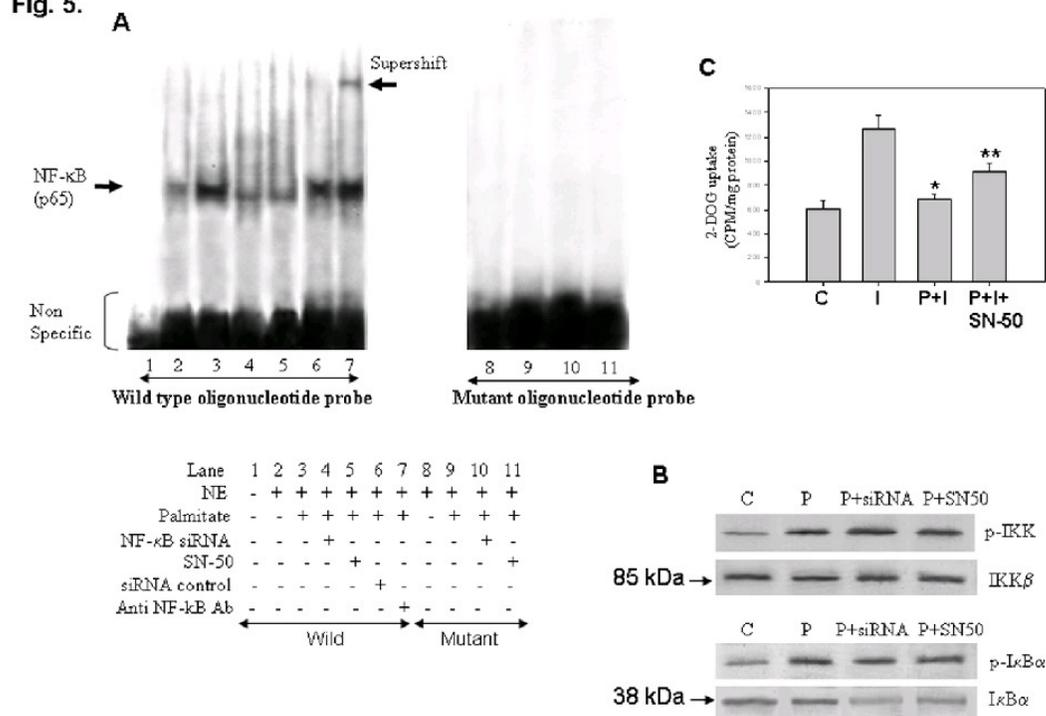


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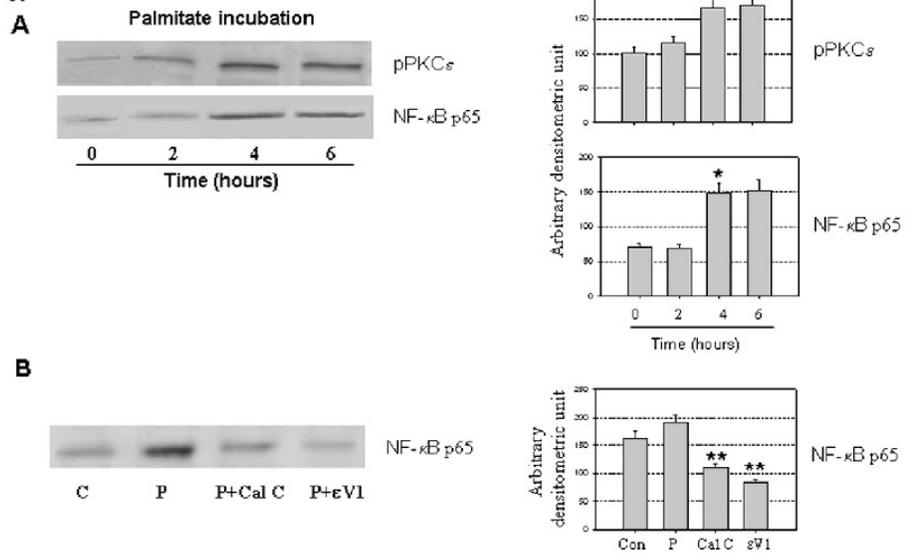
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Fig. 5.



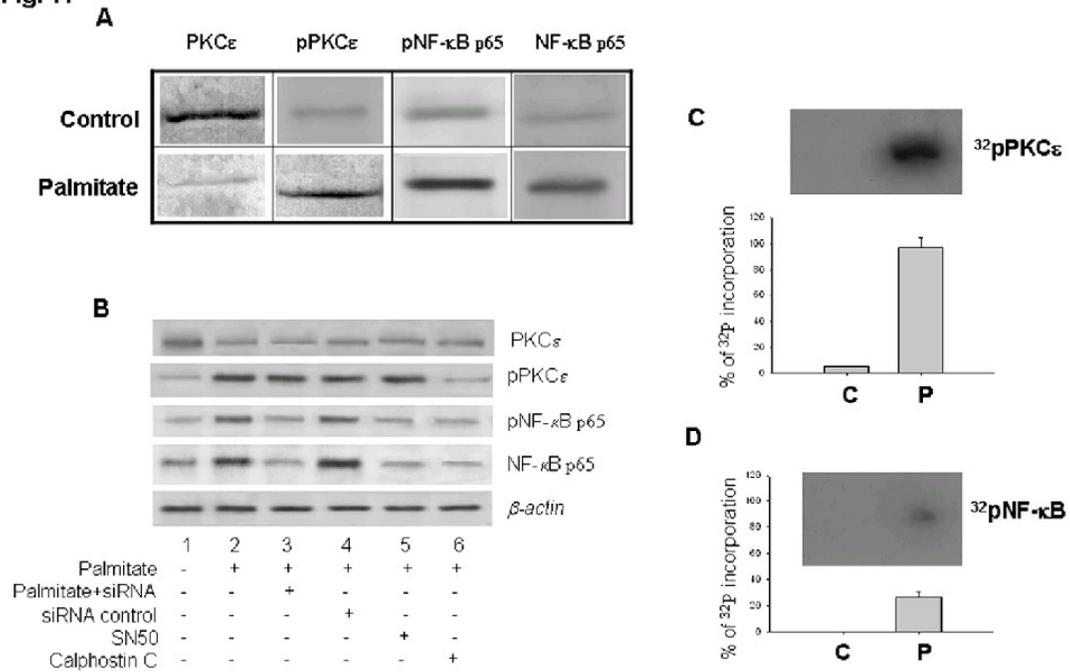
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Fig. 6.



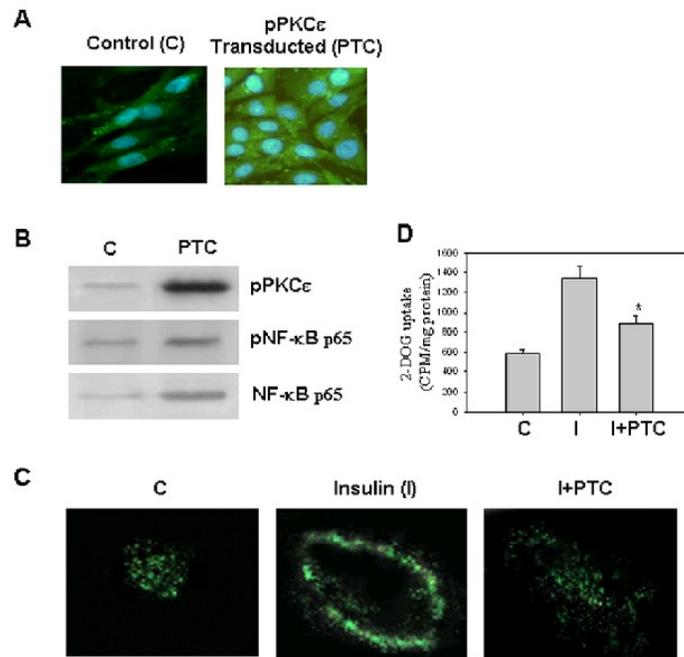
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Fig. 7.



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Fig. 9.

