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Inhibition of Insulin Receptor Gene Expression and Insulin Signaling by Fatty Acid: Interplay of PKC Isoforms Therein

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Key Words

Insulin resistance • Type 2 diabetes • Insulin receptor • Insulin signaling • PKC isoforms • Free fatty acids • HMG

Abstract

Fatty acids are known to play a key role in promoting the loss of insulin sensitivity causing insulin resistance and type 2 diabetes. However, underlying mechanism involved here is still unclear. Incubation of rat skeletal muscle cells with palmitate followed by I^{125}- insulin binding to the plasma membrane receptor preparation demonstrated a two-fold decrease in receptor occupation. In searching the cause for this reduction, we found that palmitate inhibition of insulin receptor (IR) gene expression effecting reduced amount of IR protein in skeletal muscle cells. This was followed by the inhibition of insulin-stimulated IRβ tyrosine phosphorylation that consequently resulted inhibition of insulin receptor substrate 1 (IRS 1) and IRS 1 associated phosphatidylinositol-3 kinase (PI3 Kinase), phosphoinositide dependent kinase-1 (PDK 1) phosphorylation. PDK 1 dependent phosphorylation of PKCζ and Akt/PKB were also inhibited by palmitate. Surprisingly, although PKCε phosphorylation is PDK1 dependent, palmitate effected its constitutive phosphorylation independent of PDK1. Time kinetics study showed translocation of palmitate induced phosphorylated PKCε from cell membrane to nuclear region and its possible association with the inhibition of IR gene transcription. Our study suggests one of the pathways through which fatty acid can induce insulin resistance in skeletal muscle cell.

Introduction

Insulin resistance and type 2 diabetes mellitus is an insidious disease that accounts for more than 95% of diabetic cases. This heterogeneous disorder is increasing in epidemic proportions; its worldwide frequency is expected to grow more than 6% per annum [1, 2]. The disease is primarily expressed in the form of hyperglycemia due to defects in glucose disposal into skeletal muscle, fat and liver as they become less responsive or resistant to insulin [3, 4]. Large number of

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evidences has been accumulated that hold fatty acids responsible for the loss of insulin sensitivity. Elevated fatty acid in circulation are associated with impaired insulin function and is commonly linked with obesity and type 2 diabetes [5-7]. Lowering of elevated plasma fatty acids levels normalized insulin sensitivity in diabetic subjects [8]. Incubation of isolated muscle strips or cultured muscle cells with fatty acids reduces insulin stimulated glucose uptake [6, 7, 9-11]. These reports suggest that greater deposition of lipid in insulin sensitive tissues promotes loss of its activity and insulin resistance.

How insulin loses its sensitivity and fails to act in the presence of elevated fatty acids is yet unclear. It has been observed that lowering of glucose transport by fatty acid is associated with the inhibition of insulin stimulated IRS1 and IRS1 associated PI3K phosphorylation [12, 13] that affects downstream effectors such as Akt and Glut4 [14-17]. All these suggest that impairment of insulin activity is linked to insulin signaling defects. Insulin signaling molecules that are involved in the metabolic and mitogenic actions of insulin have also been suggested to play a role in cellular insulin resistance [18-20]. Protein kinase C (PKC) isoforms represents such signaling molecules. PKC isoforms are classified as classical (cPKCα, βI, βII, γ), novel (nPKC δ,ε,θ,η) and atypical (aPKC ζ,τ/λ). cPKCs are activated by Ca^{2+} and diacylglycerol (DAG), nPKCs are activated by DAG only and aPKCs respond to neither Ca^{2+} nor DAG [21]. A few PKC isoforms are indicated to have regulatory role on insulin signaling. Expression levels and activities of some of the PKC isoforms are found to be associated with insulin resistance in type 2 diabetic patients, animal models of diabetes and different types of cell models [22-24]. Recent reports indicate a link between PKC and fatty acid-induced insulin resistance. Lipid infusion in rats and humans impaired insulin-stimulated glucose disposal in muscles and concomitant activation of certain PKC isoforms [11, 13, 25]. However, the mechanism underlying insulin-signaling defects due to fatty acid is not yet clear. In this communication we report impairment of insulin binding to insulin receptor (IR) in skeletal muscle cells by fatty acid that consequently reduced IRβ tyrosine phosphorylation and insulin induced downstream signals. In searching the cause for this impairment, we have found an inhibition of IR gene expression and an association of PKC ε with IR gene downregulation.

Materials and Methods

Materials

All tissue culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, MD 20884-9980, USA. Palmitate, trypsin, porcine insulin was purchased from Sigma chemical company, St Louis, MO, USA. 2-deoxy- [1-^{3}H] glucose (Specific activity: 12.0 Ci/mmol) was from Amersham Biosciences, USA. Calphostin C was from Biomol Research Labs Inc. (Plymouth Meeting, PA). Antibodies utilized included anti IR-β (anti-rabbit), anti-p-Tyr (anti-mouse, PY99) anti-p-IRS 1 (anti-goat, Tyr 632), anti-p-PI3K (anti-goat, Tyr 508), anti pPDK1 (anti rabbit), anti-p-Akt 1/2/3 (anti-rabbit, Ser 473), anti-p-PKC ε (anti-goat), anti-p-PKC ζ (anti-rabbit), anti-p-PKC δ (anti-rabbit) and antibodies against IRS1, PI3 K, Akt1/2, PKC ε were from Santa Cruz Biotechnology Inc., USA. Anti PDK1 (anti rabbit) was purchased from Cell Signaling Technologies, USA. Alkaline phosphatase or horse-radish peroxidase conjugated anti-rabbit, and anti-goat secondary antibodies were also purchased from Santa Cruz Biotechnology Inc., USA. All other chemicals were from Sigma Chemical Co. USA.

Cell Culture and treatments

All animals were handled in accordance with the Animal ethics committee, IICB, India. Soleus muscles from 2-3 day old neonatal Sprague-Dawley rats were dissected out and separated in a biological safety cabinet by following the method mentioned by Dennis et al [26] with some modifications. Briefly, soleus muscle strips were incubated in 0.2% collagenase and 0.05% trypsin in PBS (phosphate buffered saline 0.05M, pH 7.5, 0.15M NaCl) with continuous stirring. The dispersed skeletal muscle cells were pelleted by centrifugation at 500g, washed and resuspended in Kreb's Henseleit buffer (KHB) containing 10% fetal calf serum and 1% penicillin/streptomycin. Skeletal muscle cells were preincubated in glucose-free KHB at 37°C under 95% O_2/5% CO_2 for 1hr. Muscle cells were then seeded to collagen-coated dish containing Dulbecco's Modified Eagle's Medium (DMEM). For primary culture, beginning at 48 h of cell plating, the medium was replaced every 48h till the cells become confluent, which required 3-8 days. On attaining the confluence, cells were incubated in the absence (control) or presence of 0.75 mM palmitate (Lipid-containing media were prepared by conjugation of fatty acid with -free bovine serum albumin, by a method modified from the method described by Chavez et al. [35], with or without 100nM insulin at the time periods mentioned under different experiments. In the case of experiments with calphostin C (a strong inhibitor of protein kinase C), cells were preincubated for 30 min in 100nM calphostin C prior to incubation with fatty acid.

Radiolabeled insulin binding to solubilized receptor preparations

Membrane preparation from skeletal muscle and binding experiments were carried out by following the method previously described by us [27] with a little modification. Briefly, control and palmitate treated skeletal muscle cells were washed

thrice with 0.02M Phosphate Buffer (pH-7.4) containing 0.14M NaCl and resuspended in Lysis buffer [20mM Tris-HCl, 40mM NaCl, 5mM EDTA, 5mM Iodoacetamide, 0.1% Triton X100, pH-8.4, supplemented with protease inhibitors (1μg/ml aprotinin, 1μg/ml pepstatin, 1μg/ml leupeptin, 2mM phenyl methyl sulfonyl fluoride and 1μg/ml soybean trypsin inhibitor)], sonicated and centrifuged at 10,000 g for 15min at 4°C. The lipid-laden part of the supernatant phase was removed; supernatant was then layered on the top of a sucrose gradient formed by 50, 40, 36, and 30% sucrose (from bottom to top). Tubes were centrifuged at 60,000g in a (Sorvall Ultra-80) ultracentrifuge for 90min and the membrane fraction was obtained between 35 and 40% sucrose gradient, dialyzed overnight against 0.02M phosphate buffer (pH-8.4) and volume reduced by lyophilization. The membrane fraction was mixed with 0.1M lithium diiodosalicylate and to this solution 0.1% TritonX-100 (v/v) and 25% glycerol was added with constant stirring. The solution was dialyzed against 0.02M-phosphate buffer (pH 8.4), lyophilized to reduce the volume and used as solubilized membrane preparation. Recombinant human insulin was radiolabelled with ^{125}I and separated from free ^{125}I through Sephadex-G15 column equilibrated with 0.01M Phosphate Buffer (pH-7.2) containing 0.14M NaCl and 1% (w/v) BSA. Specific activity of ^{125}I-insulin was 30.55μCi/μg of protein.

For the preparation of solubilized receptors covalently labeled with ^{125}I-insulin, their separation through SDS-PAGE and autoradiography, we followed the method described by Velicelebi and Aiyer [28] with little modification. Briefly, solubilized membrane preparation from control or palmitate treated skeletal muscle cells (25μg from each incubation) was incubated overnight at 4°C with 2ng ^{125}I-Insulin in a final volume of 500μl of 0.02M phosphate buffer (pH-8.4) containing 0.15M NaCl and 0.25% BSA. On termination of incubation, it was pelleted by ultracentrifugation (Sorvall Ultra-80) at 10^5 g for 1hour. Pellet in each tube was dissolved in 1x sample buffer (63mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, and 0.025% Bromophenol blue) and subjected to non-reducing SDS-PAGE (4% stacking gel was layered on the top of a 7.5% resolving gel) with prestained high molecular weight protein marker (MBI Fermentas, USA). The gel was dried, exposed to Kodak X-OMAT AR for 24 h and developed.

Determination of specific binding and Scatchard analysis

Cells were incubated with or without palmitate for 8h, lysed and membranes were prepared according to the above description. To determine the optimum binding conditions of ^{125}I-Insulin to the receptor protein, binding incubations were performed at different temperatures and time intervals with varied amount of solubilized receptor preparations. It was found that overnight incubation of 15 μg of solubilized membrane protein at pH 8.4 and a temperature of 4°C permitted optimum radiolabeled insulin binding. Solubilized membrane preparations (15 μg protein) from control or palmitate treated cells were incubated overnight at 4°C in a final volume of 500 μl buffer (0.02M phosphate buffer, pH 8.4, containing 0.15M NaCl and 0.25% BSA) with varying concentrations of ^{125}I-Insulin (0.18-0.72 nmoles/L) in the absence (total binding) or presence of 10,000 fold excess unlabeled insulin (specific binding). On termination of the incubations, free and bound radioactivity was separated by adding 500 μl of 0.5% chilled polyethylene glycol (PEG; MW 6000) followed by centrifugation at 20,000g in a refrigerated centrifuge for 15 min. The supernatant was aspirated out and the pellet was washed thrice with washing buffer (0.02M phosphate buffer of pH 8.4 containing 0.15M NaCl and 0.25% BSA). The radioactivity in the final pellet was measured in a gamma counter. The specific binding was calculated by using the formula $B\text{-}b_o/B_o\text{-}b_o$ X 100 where B corresponds to the binding in the presence of cold (unlabelled insulin), b_o represents non-specific binding, and B_o was the binding in the absence of cold. Data were then subjected to Scatchard analysis to determine the affinity and capacity of insulin binding to the receptor.

Reverse transcription PCR and Northern blotting

Total RNA was extracted from control and treated skeletal muscle cells using Tri-reagent (Sigma-Aldrich). 2.5 micrograms of total RNA was reverse transcribed with MuL-V-reverse transcriptase (MBI Fermentas, Vilinius, Lithuania) primed with deoxythymidine 5'-GGAAGCTTTTTTTTTTTTTT-3' oligos. Reaction was carried out in 20μl total volume containing 10μM dithiotreitol (DTT), 0.5mM of each deoxynucleotide triphosphate, 50mM Tris-HCl (pH 8.3), 75mM KCl, and 3mM $MgCl_2$ for 1h at 42°C. Two microlitres of first strand cDNA was amplified in 50μl PCR reaction mixture containing 2.5 U Taq Polymerase (Advantage2 RT-PCR kit, BD Biosciences, Palo Alto, CA); 1.5mM $MgCl_2$; and 200μM each of deoxyNTP (Sigma - Aldrich) and the buffer supplied with the enzyme along with 2μl each of sense and antisense primers diluted to 10μM. Following primers were designed –

IR sense, 5'-GGATGGTCAGTGTGTGGAGA-3'
antisense, 5'-TCGTGAGGTTGTGCTTGTTC-3';
β actin sense,
5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'
antisense,
5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'.

Insulin receptor sequence from *Rattus norvegicus* (GeneID**:** 24954) was chosen for designing the primers for insulin receptor gene, and amplifying approximately 165 bp segment of rat insulin receptor homologue. The PCR was performed for 30 cycles of denaturation at 94°C, for 45sec(5 min in the first cycle), annealing at 55°C for 30sec, and extension at 72°C for 1 min (10 min last cycle; Perkin Elmer 9700; PE Applied Biosystems, Foster City, CA) with a manual hotstart protocol. A house keeping gene β actin was amplified simultaneously to serve as an internal control. The PCR product of insulin receptor was cloned into pTZ57R/T plasmid using InsTA clone PCR cloning kit (MBI Fermentas) following the manufacturers instruction and sequenced using ABI PRISM 3100 (Perkin Elmer PE, Applied Biosystems) automated sequencer. Sequence analysis was done by BLAST software available on the NCBI Website.

RNA was isolated from skeletal muscle cells using the Tri reagent RNA extraction kit (Sigma Chemical Co., USA) following manufacturer's instructions. 10 µg of total RNA were loaded on each lane of a 1% formaldehyde-agarose gel electrophoresed and transferred to a PVDF membrane. The membrane was hybridized with XbaI-BamHI fragment of rat insulin receptor cDNA cloned in TA cloning vector. Prehybridisation was allowed for 4h in the buffer containing 6x SSC with 80% formaldehyde, 1x Denhardt's solution and 0.5% SDS at 42°C. Hybridization was carried out under similar conditions with γp^{32}-labelled rat insulin receptor cDNA for 12h. The membrane was washed for 90min at 65°C in 2x SSC containing 0.1% SDS with subsequent three changes of buffer. The hybridized membrane was exposed to Kodak X-ray film for 24h (Eastman Kodak Company, Rochester, NY) at 80°C. The digestion of DNA, agarose gel electrophoresis, DNA random labeling and Northern hybridization were performed according to the method described by Sambrook et al.

Electrophoresis and Immunoblotting

Cell pellets from control and treated incubations were suspended 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 at 150 KHz for 5 min. Lysates were centrifuged for 10 min at 10,000g, supernatant was collected and protein content was estimated according to Lowry *et al* [29]. Control and treated cell lysates (50 µg) 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. Electrophoresis was carried out at 90V constant voltage. Membranes were incubated with 5% Blocking buffer (20mM Tris base, 137mM NaCl, 1mM HCl, 0.1% Tween 20 and 5% non-fat milk) for 1h followed by incubation with primary antibodies such as anti insulin receptor β (IRβ), anti p-IRS 1 (anti-goat; 1:1000), anti p-PI3K (anti-goat; 1:1000) and anti p-Akt 1/2/3 (anti-rabbit; 1:1000), anti-p-PKC ε (anti-goat), anti-p-PKC ζ (anti-rabbit), anti-p-PKC δ (anti-rabbit) antibodies, overnight at 4°C. A concurrently run gel was immunoblotted with anti IRS 1(anti-goat; 1:1000), anti PI3K (anti-goat; 1:1000), anti PDK1 (anti rabbit;1:1000), anti PKC ε (anti goat; 1:1000) and anti Akt 1/2 (anti-rabbit; 1:1000) antibodies. Bound primary antibodies were visualized using corresponding secondary antibodies at 1:1000 dilution, which were tagged either with alkaline phosphatase or horse-radish peroxidase and were developed with corresponding substrates nitro blue tetrazolium / 5-bromo 4-chloro 3-indolyl phosphate or tetramethyl benzidene / H_2O_2 respectively.

Immunoprecipitation

To observe insulin receptor β tyrosine phosphorylation, control and palmitate treated skeletal muscle cells were incubated with insulin (100 nM) for 30 min, lysed by sonication in lysis buffer (as described above) followed by centrifugation at 10,000 g for 10 min. Supernatant was collected, and 200 µg of protein was incubated overnight at 4°C with 2 µg insulin receptor β (IR β) antibody. 50 µl of protein A-sepharose was then added to each tube and incubated at 4°C for 2h followed by centrifugation at 10,000g. Immune-complexed IR β was resuspended in 500 µl of 0.1% CHAPS in PBS, washed thoroughly and subjected to SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody (anti-mouse; 1:1000).

Immunofluorescence staining

Skeletal muscle cells were seeded on sterile uncoated 22-mm coverslips and grown for 2 days in DMEM with 10% fetal bovine serum in a humidified atmosphere of 95% O_2/ 5% CO_2 at 37°C. Cells were serum starved for 10h and incubated with palmitate (0.75mM) at different time periods, control incubation contained BSA as vehicle. On termination of incubation, cells were subjected to fixation with 3.5% paraformaldehyde in Hanks' salt solution containing 20 mM HEPES, pH 7.0, permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and 1% BSA for 5 min followed by incubation with anti-PKCε antibody. After treatment with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100), coverslips were mounted in glycerol/PBS and viewed with fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) where the F value indicated significance, means were compared by Duncun's multiple range post hoc test. All values were means ± SEM of three independent observations.

Results

Downregulation of IR and IR gene expression by palmitate

Skeletal muscle cells were incubated in the absence (control) or presence of palmitate for 8h. Plasma membrane preparation from them was solubilized and allowed to bind with radiolabelled insulin. Proteins were then resolved through non-reducing PAGE followed by autoradiography. Fig 1a shows that ^{125}I was bound to 210 kDa protein which corroborated earlier reports on detergent solubilized IR [28, 30, 31]. ^{125}I-insulin binding to IR was greatly reduced due to palmitate incubation. In a separate experiment, membrane preparation from control or palmitate-incubated skeletal muscle cells was subjected to in-vitro binding incubation with ^{125}I-insulin in the presence or absence of unlabeled insulin. Palmitate did not alter K_a but it effectively reduced B_{max} from 7.3pM to 3.46pM (Fig 1b). Unaltered K_a indicate that inhibition by palmitate is not due to modulation of IR protein, it is possibly for the reduction in IR number since B_{max} gets reduced. This led us to determine the expression of IR

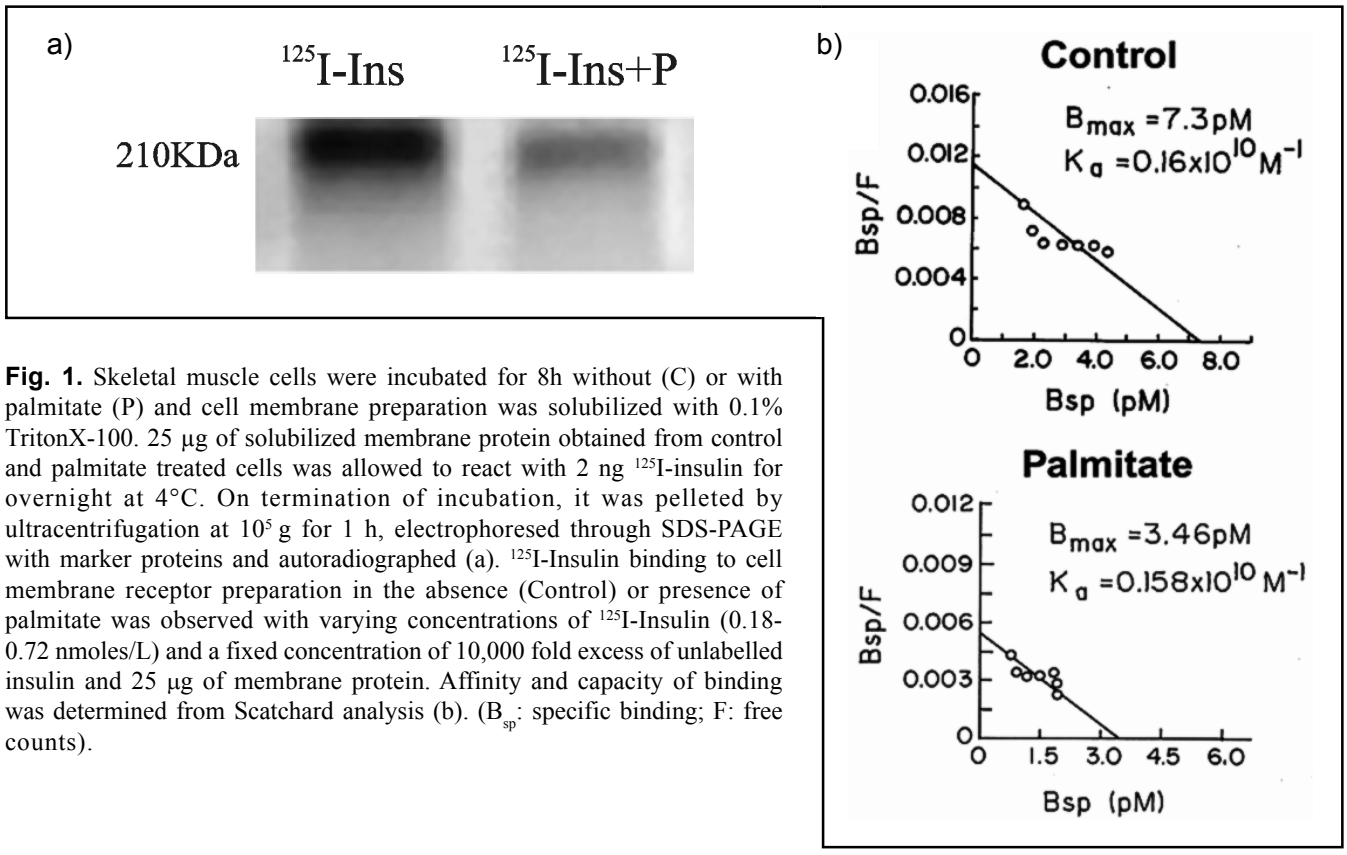


Fig. 1. Skeletal muscle cells were incubated for 8h without (C) or with palmitate (P) and cell membrane preparation was solubilized with 0.1% TritonX-100. 25 μg of solubilized membrane protein obtained from control and palmitate treated cells was allowed to react with 2 ng ^{125}I-insulin for overnight at 4°C. On termination of incubation, it was pelleted by ultracentrifugation at 10^5 g for 1 h, electrophoresed through SDS-PAGE with marker proteins and autoradiographed (a). ^{125}I-Insulin binding to cell membrane receptor preparation in the absence (Control) or presence of palmitate was observed with varying concentrations of ^{125}I-Insulin (0.18-0.72 nmoles/L) and a fixed concentration of 10,000 fold excess of unlabelled insulin and 25 μg of membrane protein. Affinity and capacity of binding was determined from Scatchard analysis (b). (B_{sp}: specific binding; F: free counts).

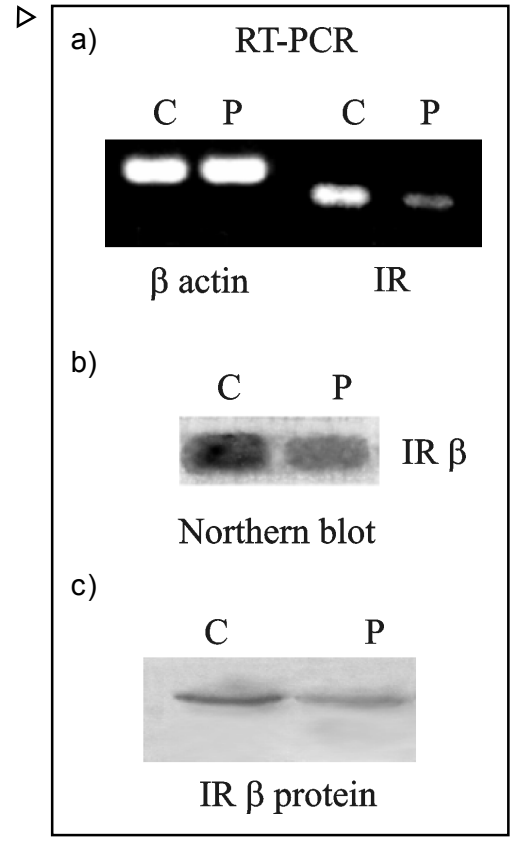


Fig. 2. Expression patterns of IR gene was examined by semiquantitative RT-PCR using RNA samples from vehicle (C) or palmitate treated (P) skeletal muscle cells, β actin was used as an internal control (a). Determination of palmitate induced reduction of IR gene expression by Northern blotting. 10 μg each of total RNA from control and palmitate treated skeletal muscle were electrophoresed and transferred onto a nylon membrane (immobilon-Ny+, Millipore, Bedford, MA). The membrane was then hybridized with insulin receptor cDNA (b). Skeletal muscle cells were treated without (C) or with palmitate and then incubated with insulin for 30 min. On termination of incubations, cells were lysed by sonication and 50μg protein from each cell lysate was resolved in SDS-PAGE and then subjected to Western blot analysis with anti insulin receptor β (IRβ) antibody for determination of the IRβ protein content (c).

gene and semiquantitative PCR data showed a significant decrease in IR mRNA in palmitate treated muscle cells (Fig.2a). To have additional data on IR gene expression we performed Northern hybridization by using rat insulin receptor cDNA as a radiolabelled probe. Palmitate incubations strongly reduced IR mRNA expression as compared to control (Fig.2b). Western blot analysis shows that the protein level also decreases consequently in palmitate incubated cells as compared to the control (Fig 2c). These results suggest that fatty acid -induced insulin resistance is related to reduced expression of IR gene.

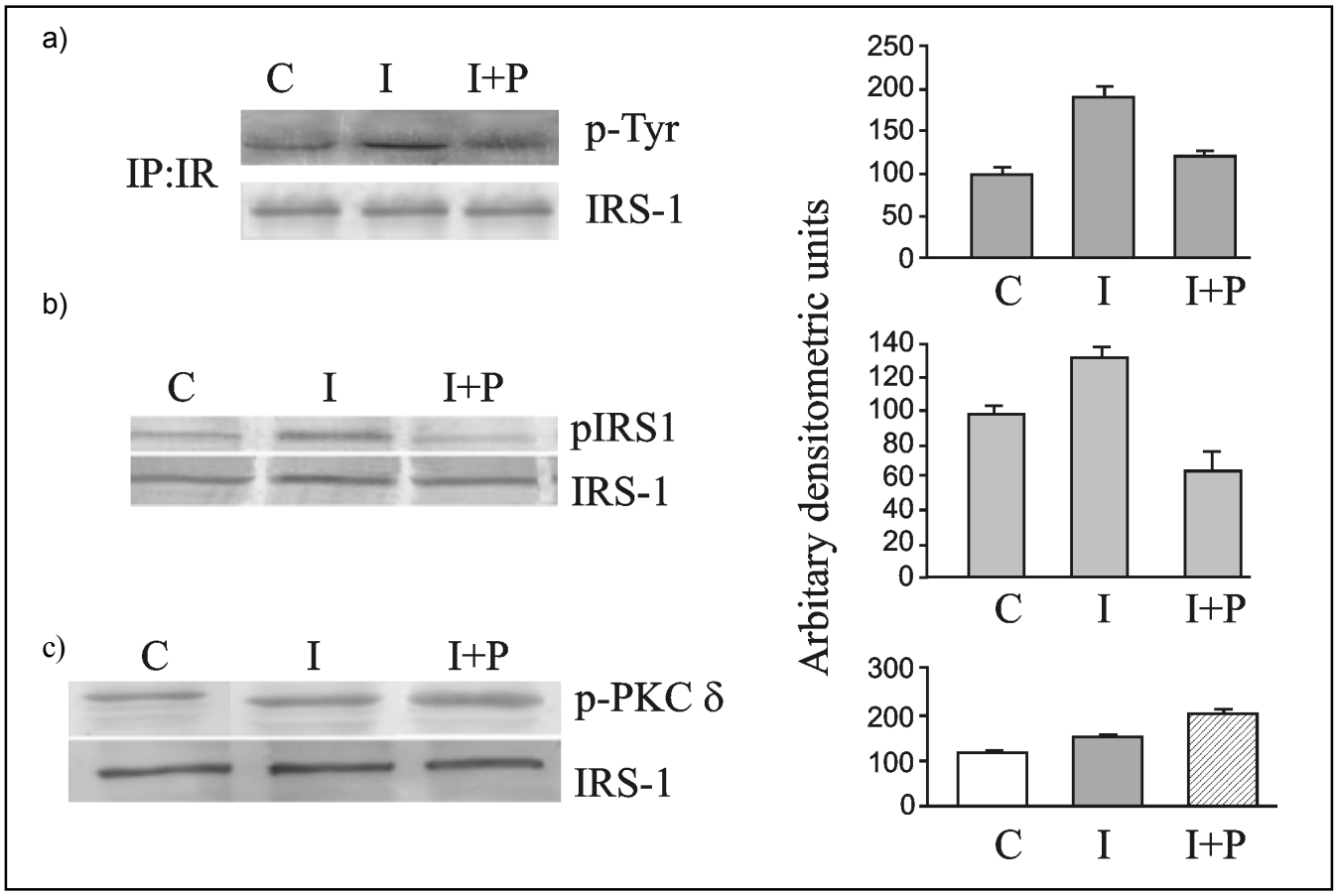


Fig. 3. Control or palmitate incubated skeletal muscles were treated with insulin for 30 min, lysed by sonication in lysis buffer and centrifuged for 10 min at 10,000 g. 200 µg supernatant protein from control and treated cells was incubated overnight at 4°C with 2 µg IRβ antibody. The antigen-antibody complex was pelleted with protein A-agarose, immunoprecipitated pellets were washed thoroughly, resuspended in SDS-PAGE sample buffer, boiled and electrophoresed. Proteins from the gel were transferred to PVDF membrane and immunoblotted with anti-p-Tyr antibody. C-control; I-Insulin; I+P-insulin plus palmitate. IRS-1 was used as a loading control (a). Skeletal muscle cells were treated without (C) or with palmitate for 8 h. and then incubated with insulin for 30 min. On termination of incubations, cells were lysed by sonication and 50 µg protein from each cell lysate was resolved in SDS-PAGE and then subjected to Western blot analysis with phospho-specific antibodies against pIRS1 (1:1000) (b) or pPKCδ (1:1000) (c). IRS 1 was used as the loading control. Densitometric analysis of immunoblots was performed by Image J software.

Involvment of PKC isoforms in fatty acid induced inhibition of insulin signals

Insulin binding to the receptor results in phosphorylation of IRβ tyrosine kinase. When solubilized receptor preparation from control or palmitate incubated muscle cells were allowed to form immuno-complex with anti-IRβ antibody and subsequently probed with anti-phosphotyrosine antibody, a significant decrease in insulin-stimulated tyrosine phosphorylation of IRβ was observed in the receptor preparation from palmitate incubated cells (Fig.3a). Results indicate a correlation between palmitate-induced inhibition of IR gene expression, insulin-IR complex and insulin receptor tyrosine kinase phosphorylation. This prompted us to examine insulin-stimulated downstream signals, which are consequently phosphorylated following IRβ tyrosine kinase phosphorylation. Insulin stimulated IRβ phosphorylation follows IRS 1 tyrosine phosphorylation and that was clearly inhibited by palmitate (Fig. 3b). A PKC isotype, PKCδ, was implicated in IR regulation. Palmitate treated skeletal muscle cells showed greater phosphorylation of PKCδ as compared to insulin alone (Fig. 3c), suggesting involvement of PKCδ in the inhibition of IRβ and IRS1 tyrosine phosphorylation. We then investigated the effect

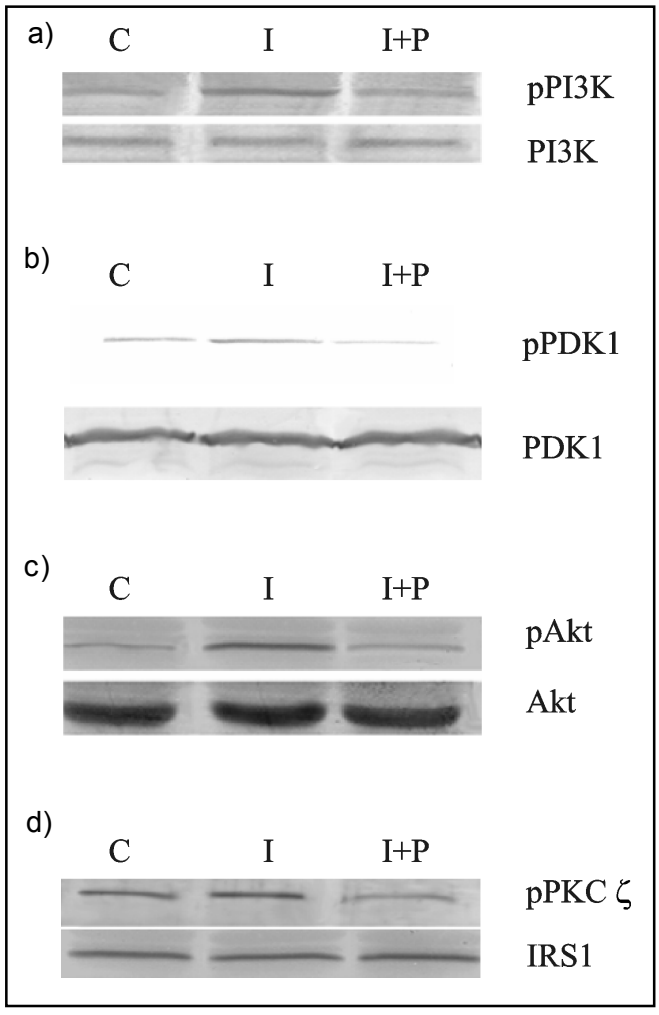


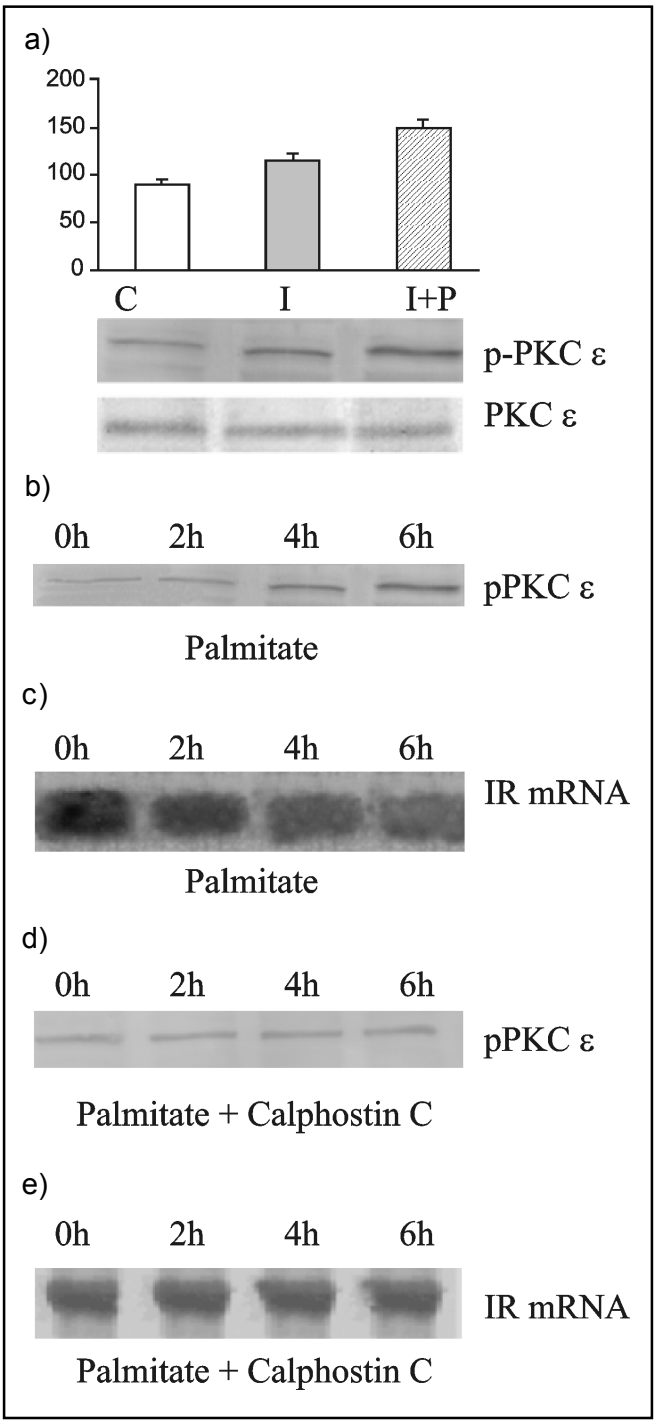
Fig. 4. Skeletal muscle cells were incubated in the absence of insulin and palmitate and that was taken as control (C). Control cells exposed to insulin for 30 min (I) or palmitate incubated cells for 8h and then incubated with insulin for 30 min (I+P). On termination of incubations, cells were lysed by sonication and 50 µg protein from each cell lysate was resolved in 10% SDS-PAGE and then subjected to Western blot analysis with phospho-specific antibodies against pPI3 Kinase (1:1000) (a), pPDK1 (1:1000)(b) or pAkt 1/2 (1:1000) (c) and PKCζ (d). In a separate set of experiment immunoblotting was performed with corresponding non-phosphorylated antibodies for PI3 kinase, PDK1 and Akt 1/2, for PKC ζ anti IRS 1 was used as loading control.

of palmitate on IRS1 associated PI3 kinase phosphorylation. Insulin-stimulated phosphorylation of PI3 kinase is expected to be inhibited by palmitate as it causes reduction in IRS1 phosphorylation. Fig. 4a and 4b demonstrate inhibition in insulin-stimulated phosphorylation of PI3 kinase and PI3 kinase dependent protein kinase 1 (PDK1) by palmitate. In insulin signal transduction pathway, PDK1 is the upstream kinase that directly phosphorylates downstream substrates such as Akt/PKB and PKC ζ. Insulin stimulated phosphorylation of Akt/PKB and PKC ζ was inhibited in palmitate incubated skeletal muscle cells (Fig. 4c and 4d). Palmitate did not change PI3 kinase, PDK1, and Akt protein profile of skeletal muscle cells.

Induced phosphorylation of PKC ε is related to reduced IR gene expression

Although above description of our results clearly indicate palmitate induced inhibition of insulin signaling pathway and involvement of PKC δ and PKC ζ there on, it could not suggest the possible mechanism involved in the inhibition of IR gene expression by palmitate. Phosphorylation of PKCε is dependent on PDK1, PKCε becomes a substrate of PDK1 when it is membrane bound. We had an unexpected result; palmitate incubation of skeletal muscle cells intensified insulin stimulated phosphorylation of PKCε. Since palmitate inhibited PDK1 phosphorylation, one would expect inhibition of PKCε by it. In contrast, palmitate stimulated phosphorylation of PKCε was more than what was observed with insulin (Fig 5a), pamitate did not alter PKCε protein content. Surprisingly, incubation of muscle cells with palmitate alone increased PKCε phosphorylation. In searching the possible cause for this, we found an earlier report describing constitutive phosphorylation of PKCε by fatty acid in a PDK1 independent way [32]. Considering this to be a probability, we conducted a time kinetic study that showed an interesting trend. Palmitate stimulated PKC ε phosphorylation in a time dependent manner, increase of phosphorylation was detected at 2h which increased further at 6h (Fig 5b). Activation of PKC ε was inversely related to IR gene expression, palmitate decreases IR mRNA in a similar time dependent manner (Fig 5c). Calphostin C, an inhibitor of PKC, blocked palmitate induced PKC ε phosphorylation (Fig 5d). Palmitate could not reduce IR gene expression in the presence of calphostin C (Fig 5e). This indicates an association of activated PKC ε with reduced IR gene expression. Fig 6 demonstrates that change in the localization of PKC ε occurs due to phosphorylation by palmitate, at 2h phosphorylated PKCε was detected in the plasma membrane fraction, while at 6h most of the phosphorylated PKCε was associated with nuclear fraction (Fig 6a). Detection with immunofluorescent probe also showed translocation of pPKCε from cytosol to nuclear region at 6h (Fig 6b). Hence, experiments with palmitate showed

Fig. 5. Skeletal muscle cells were treated without or with palmitate for 8h and insulin was added to both control (I) and palmitate (I+P) incubated cells. Cells without any of the treatments were taken as control (C). On termination of incubations, cells were lysed by sonication and 50 µg protein from each cell lysate was resolved in 10% SDS-PAGE and then subjected to Western blot analysis with anti PKCε or anti pPKCε antibodies (1:1000) (a). A time kinetic study was carried out by incubating cells with palmitate at different time periods. At the end of each incubation, cells were similarly lysed, subjected to SDS PAGE and was immunoblotted against anti pPKCε antibody (anti Goat, 1:1000) (b). A Northern blot analysis showing time dependent decrease of IR mRNA (c). Western blot of pPKC ε demonstrating inhibition of palmitate stimulated phosphorylation of PKC ε by Calphostin C (100nM) (d). Northern blot shows unaltered IR mRNA level in the presence of Calphostin C (e).



a time dependent translocation of phosphorylated PKCε indicating a possible involvement of PKC ε in palmitate induced inhibition of IR gene transcription.

Discussion

Several studies suggest oversupply of lipid to peripheral tissues is responsible for the development and severity of insulin resistance. Patients suffering from insulin resistance frequently display signs of abnormal lipid metabolism, increased circulatory concentrations and elevated deposition of lipid in skeletal muscle [9, 33]. It is now well accepted that fatty acid opposes insulin action but how it does so still remains unclear. We have observed impairment of insulin binding to its receptor due to palmitate. Our selection of palmitate as a fatty acid was based on our experiments with different saturated and unsaturated fatty acids (data not shown) and also earlier reports where it was shown to be the most potential fatty acid in impairing insulin sensitivity [34-37]. It was not clear to us how palmitate incubation could inhibit insulin binding to IR; since there was no change in K_a while B_{max} was reduced to two-fold, a reduction in IR number appeared to be related to this inhibition. This led us to investigate whether this downregulation of IR number is due to the impairment of IR gene expression causing reduction in IR protein. Both RT-PCR data and Northern analysis demonstrated an inhibition of IR gene expression by palmitate. The mechanism underlying here is unknown; however our study on PKC isoforms indicates a possible pathway.

The insulin receptor is a heterotetrameric tyrosine kinase receptor, binding of insulin activates its kinase and that phosphorylates intracellular docking proteins IRS1, IRS2. These docking molecules then recruit and stimulate various effector enzymes [34, 38]. IRS proteins phosphorylate PI3Kinase, which in turn converts

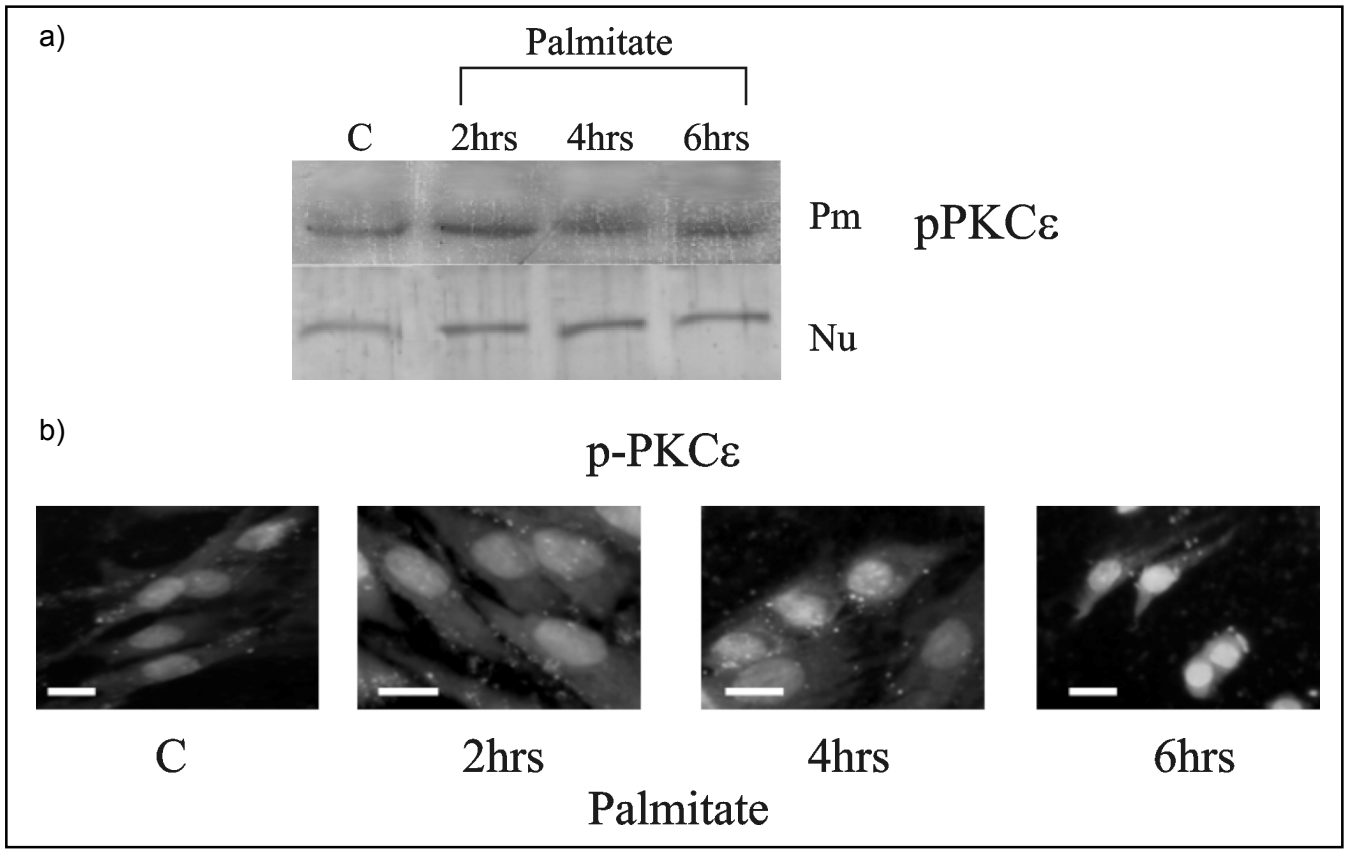


Fig. 6. Translocation of palmitate induced pPKCε was observed at different time periods (2h, 4h, 6h). Plasma membrane (pm) and nuclear fractions (nu) from control (C) or palmitate treated cells were obtained by following the procedures described by Frevert and Kahn, 1996 [45]. Proteins were separated by 10% SDS PAGE and then immunoblotted with anti pPKCε antibody (a). Distribution of palmitate induced phosphorylated PKCε in skeletal muscle cells as revealed by fluorescence microscopy. Skeletal muscle cells were seeded on to collagen-coated coverslips and incubated without (C) or with palmitate at different time periods, control incubation contained BSA as vehicle. After fixation with paraformaldehyde they were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and 1% BSA followed by incubation with anti-PKCε primary antibody (1:100) and fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:100) the coverslips were mounted in glycerol/PBS and viewed under a fluorescence microscope (b). Bars correspond to 10 μm.

phosphoinositides to phosphatidylinositol 3,4,5-trisphosphate that recruits PDK1 and PDK1 dependent cytosolic Akt/protein kinase B (PKB) to the plasma membrane. PDK1 promotes Akt/PKB activation by facilitating phosphorylation of its regulatory domains [39]. Activated Akt/PKB effected translocation of Glucose transporter 4 (Glut4) to the cell surface membrane [40]. We have found an inhibition of IRβ tyrosine phosphorylation in palmitate incubated muscle cells. This is expected as palmitate-inhibited IR gene expression will decrease the amount of Insulin-IR complex and that will reduce IRβ tyrosine phosphorylation. Moreover, we tracked every step of insulin signaling starting from the inhibition of IR to IRS1, PI3 kinase, PDK1 and Akt/PKB phosphorylations by palmitate so that the critical point in fatty acid induced impairment of insulin sensitivity could be identified. From our observations, inhibition of IR gene expression appears to be the most important point.

The molecular mechanisms of insulin resistance are indeed very complex, on the cellular level insulin resistance may occur at multiple steps of the insulin-signaling pathway. Among the most important candidates, recent studies implicate PKC isoforms as major players. These reports focused on the role of nPKCs, PKC δ, PKC θ and PKC ε in fatty acid-induced insulin resistance [11, 20, 25]. nPKCs are found to be activated in muscles from hyperinsulinemic and hyperglycemic humans and rats [41, 42] suggesting their involvement in the impairment of

insulin sensitivity. Consistent with these studies is the observation that PKC δ mediated inhibition of IR kinase-catalyzed IRS1 tyrosine phosphorylation occurs as a consequence of direct Ser/Thr phosphorylation of IRS1 by PKC δ [20]. PKC δ has also been implicated in the internalization of IR thus attenuating insulin-induced tyrosine phosphorylation of IR [43]. Our time kinetic data on PKCδ phosphorylation and IR mRNA expression does not indicate its link to fatty acid induced IR gene downregulation. Palmitate induced inhibition of IR gene expression has been detected at 2 h which further decreased at 4h and 6h, whereas inhibition of PKC δ could only be detected at 6 h and not earlier. This indicates that PKC δ is not involved in the palmitate induced downregulation of IR gene expression. On the other hand, PKC δ here may be involved in the inhibition of IR kinase activity as observed by Greene et al [20] or internalization of IR as shown by Braiman et al [43]. One of the atypical PKCs, PKC ζ has been shown to be activated by insulin through PI3 kinase and PDK1 dependent pathway [44]. The association between PDK1 and PKC ζ appears to be very close and there is extensive cross talk between them [45, 46]. We have found that inhibition of PDK1 phosphorylation is reflected on reduced PKC ζ activity suggesting close association of these kinases in the PI3 kinase-signaling pathway.

The novel PKCε isotype has recently been shown to be linked to insulin signaling pathway. Activation loop of PKCε is directly phosphorylated by PDK1 and therefore it is another kinase in the PI3K-PDK1 dependent signaling. It has been suggested that overexpression and chronic activation of PKCε is related to the development of insulin resistance in animals [47]. In vitro studies indicated that PKCε plays an important role in mediating the inhibitory effect of TNFα on insulin signaling; TNFα is the major cause of obesity linked insulin resistance [48, 49]. PKCε has recently been implicated in the downregulation of IR. Ikeda et al., [47] reported that reduction in IR numbers in the skeletal muscles of diabetic *P. obesus* is correlated with the overexpression of PKCε, this may be a possible mechanism for inducing insulin resistance. Our investigation showed fatty acid induced downregulation of rat skeletal muscle IR molecule is related to inhibition of IR gene expression. We then observed a consequent stimulation of PKCε phosphorylation by palmitate and translocation of PKCε from plasma membrane to nuclear region. Since phosphorylation of PKC ε is PDK1 dependent, we were puzzled to find inhibition of PDK1 phosphorylation by palmitate but remarkable increase in PKC ε phosphorylation at the same time. A report by Cenni et al [32] cleared our confusion. They demonstrated that myristoylation of PKCε resulted constitutive phosphorylation at Thr 566 and Ser 729 in the kinase domain required for PKCε activity. This phosphorylation was independent of PDK1. Palmitoylation of PKB similarly increased its activity, which could not be stimulated further by growth factors [50]. In the same way palmitoylation in our investigation probably caused constitutive phosphorylation of PKCε effecting its translocation to nuclear region. Mobilization of fluorescence-tagged pPKCε also indicated the same. These findings suggest a relationship between PKCε and IR mRNA reduction. A similar kind of observation has been made by Ikeda et al. [47], they found a significant inverse correlation between insulin binding to IR and PKCε expression. Role of PKCε in regulating LDL receptor gene transcription has recently been shown in human hepatoma cell line [51]. We have observed that palmitate activation of PKC ε occurs at 2h that increased substantially at 6h which is inversely related to IR gene transcription, as considerable inhibition of IR mRNA could be observed at 6 h by palmitate. Moreover, Calphostin C inhibition of palmitate stimulated PKCε phosphorylation is coincided with the withdrawal of IR mRNA inhibition. These data therefore indicate an association of PKCε in palmitate induced downregulation of IR gene expression. However, underlying mechanism of this is not known. There is a possibility that activated PKCε phosphorylates HMGA1 (a high mobility group protein); an integral molecule of IR transcription factor complex required for IR gene transcription [52]. It has been shown that phosphorylation blocks HMGA1 mobilization to promoter region of the gene [53]. Moreover, inhibition of HMGA1 migration by PKC was reported earlier [54, 55]. Hence, from previous reports along with our present observations, it appears reasonable to interpret that translocation of palmitate activated PKCε to nuclear region might be affecting integration of IR transcription factors due to inhibition of HMGA1 mobilization. Further investigation is required to explain the mechanism involved in IR gene transcription by PKC ε. Present observations suggest one of the possible pathways in fatty acid induced insulin resistance.

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