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Developmentally spliced PKC β II provides a possible link between mTORC2 and Akt kinase to regulate 3T3-L1 adipocyte insulin-stimulated glucose transport

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Abstract

Functional adipocyte glucose disposal is a key component of global glucose homeostasis. PKC β II is involved in rat skeletal muscle cell ISGT. Western blot analysis and Real-Time PCR revealed 3T3-L1 cells developmentally regulated PKC β splicing such that PKC β I was downregulated and PKC β II was upregulated during the course of differentiation. An initial glucose uptake screen using PKC inhibitor LY379196 pointed to a PKC isozyme other than PKC ζ mediating 3T3-L1 adipocyte ISGT. Subsequent use of PKC β II inhibitor CGP53353 pointed to a role for PKC β II in ISGT. Western blot analysis showed that CGP53353 specifically inhibited phosphorylation of PKC β II Serine 660. Subcellular fractionation and immunofluorescence demonstrated that PKC β II regulates GLUT4 translocation. Further western blot, immunofluorescence and co-immunoprecipitation analysis reveal that PKC β II inhibition does not affect mTORC2 activity yet abrogates phosphorylation of Akt Serine 473. PKC β II regulates GLUT4 translocation by regulating Akt phosphorylation and thus activity.

Keywords

PKC β II; GLUT4; Akt; mTORC2

Introduction

The 3T3-L1 cell line is the model of choice when studying fat cell development and signaling since results in 3T3-L1 adipocytes have repeatedly been confirmed in mouse models [1]. During differentiation, many genes are programmed to initiate or cease. cDNA microarray analysis of 3T3-L1 cells show PKC β expression at least ten fold higher in 3T3-L1 adipocytes versus 3T3-L1 fibroblasts [2]. This suggests a role for PKC β in adipogenesis and other adipocyte metabolic functions.

Protein Kinase C (PKC) is a family of 11 different serine/threonine kinases, and their respective splice variants, that are implicated in wide range of G protein-coupled receptor

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and other growth factor-dependent cellular processes [3]. PKCs diverge into three groups contingent on cofactor requirements. Classical PKCs (α , β I, β II, γ) require diacylglycerol, phospholipid and Ca^{2+} for full activity. PKC β I and PKC β II are encoded by the same gene but translated from alternatively spliced products of PKC β pre-mRNA. Inclusion of the PKC β II exon in the V5 region through alternative splicing results in the PKC β II mRNA [4].

PKC β II has a more prominent role in glucose uptake than PKC β I [5;6;7]. Acute insulin treatment of L6 rat skeletal muscle causes a switch from constitutive splicing (PKC β I isoform) to alternative splicing (PKC β II isoform) via phosphoinositide-3-kinase (PI3K)/Akt-mediated signaling [7]. PKC β promoter dysregulation is linked to decreased PKC β II levels resulting in insulin resistance in humans [8]. Dysregulation of PKC β alternative splicing is likely related with the pre-diabetic state. This warrants examination of the role of PKC β signaling.

Like skeletal muscle, adipocyte glucose uptake occurs via insulin-stimulated Glucose Transporter 4 (GLUT4) translocation from intracellular storage sites to the plasma membrane (PM). Global glucose homeostasis via adipocytes is not only based upon increased or decreased adiposity but on the GLUT4 signaling inside the adipocyte [9]. GLUT4 signaling has effects on circulating serum adiponectin, an adipokine crucial for peripheral insulin sensitivity [10]. Adipose-specific overexpression of GLUT4 has been reported to reverse insulin resistance and diabetes in mice lacking muscular GLUT4 [11]. Adipose-selective targeting of the GLUT4 gene in mice impairs insulin action in muscle and liver [12]. Regulation of adipocyte GLUT4 affects not only adipocyte glucose uptake but global glucose homeostasis.

Atypical PKC ζ and PKC λ have been the most extensively studied PKCs in adipocyte glucose uptake [13]. The role that PKC β II plays in insulin-stimulated adipocyte glucose regulation is unclear. The aim of this study was to determine when PKC β II expression occurred during adipogenesis and elucidate a role for PKC β II in adipocyte ISGT.

Materials and Methods

Cell Culture: Mouse 3T3-L1 pre-adipocytes obtained from American Type Tissue Culture repository, ATCC (Manassas, VA) were maintained and passaged as pre-confluent cultures in DMEM high glucose (HG) (Invitrogen, Carlsbad, CA) with 10% newborn calf serum (Sigma-Aldrich, St. Louis, MO) at 37°C and 10% CO₂. Once confluent, cells were differentiated (day 0) in DMEM HG with 10% fetal bovine serum (Atlas Biological, Fort Collins, CO), 10 μ g/mL bovine insulin (Sigma), 1mM dexamethasone (Sigma), and 0.5mM isobutyl-1-methylxanthine (Sigma). On day 2, media was replaced with DMEM HG, 10% FBS, and bovine insulin. Day 4 and afterwards, cells were cultured in DMEM HG plus 10% FBS.

Western Blot Analysis: was performed as previously described [6]. Antibodies listed in Supplemental Material.

Real-Time PCR: Total RNA was extracted using RNA Bee (Tel Test Inc., Friendswood, TX). 2 μ g RNA was used for reverse transcription using Omniscript RT kit (Qiagen, Valencia, CA, #205113) according to manufacturer's protocol. Real-Time PCR reactions were performed using TaqMan Universal PCR MasterMix (Applied Biosystems (AB) Inc., Foster City, CA, #4304437) according to manufacturer's protocol. Primers and probes are listed in Supplemental Material.

Glucose Uptake: [³H]2-deoxyglucose uptake was measured in six-well plates according to published protocols [14]. Briefly, 3T3-L1 adipocytes were serum starved with DMEM HG

for 4 h prior to treatment. Medium was then changed to 1mL Krebs-Ringer HEPES (KRH) $\pm$ CGP53353 (Novartis, Basel, Switzerland) or $\pm$ LY379196 (Eli Lilly and Company, Indianapolis, Indiana, USA) for 30 min then $\pm$ 100nM pig insulin (Sigma) 15 min.

Subcellular fractionation: was performed as described by Elmendorf et al. [15]. Three 25cm plates of cells were used per condition. Protein levels in each fraction were quantified using the BCA protein assay (Pierce).

PM Sheet Assay: 3T3-L1 cells were differentiated to day 8 on BD BioCoat Collagen Type I 8-well CultureSlides. PM sheets were obtained using a modified protocol of Olson et al. [16]. Specifics can be found in Supplemental Material.

Immunofluorescence measure of pAkt Ser473: Immunofluorescence protocol is detailed in Supplemental Materials.

Co-Immunoprecipitation: was performed using Protein A Magnetic Beads #S1425S (New England Biolabs, NEB, Ipswich, MA) according to manufacturer's protocol. Antibodies are listed in Supplemental Material.

Results

Developmentally regulated alternative splicing of PKC β in 3T3-L1 adipocytes

During adipogenesis PKC β expression increases in 3T3-L1 adipocytes [2]. Whether this reflects PKC β I or a switch to PKC β II was unknown. By following 3T3-L1 cells through differentiation, we found PKC β I was initially expressed on day 0 (Fig. 1A). As differentiation proceeded, PKC β I expression declined while PKC β II expression increased dramatically, peaking around day 8 (Fig. 1A-C). PPAR γ , adiponectin and GLUT4 are markers of adipocyte differentiation. PKC β II mRNA expression mimicked protein expression with a peak at around day 6 (Figure 1D). From day 0 to day 6 there is almost a 13-fold increase in PKC β II mRNA expression. This data is in agreement with reports showing PKC β expression increasing at least 10-fold from pre-adipocyte to adipocyte in microarray analysis [2]. However, a switch in isoform expression from PKC β I to PKC β II was not reported. Possible factors responsible for the shift in PKC β spliced isoforms were the focus of initial experiments and are reported as supplemental data (Supplemental Figure (SF) 1A-B). The differentiation pattern of PKC isoforms α , γ , δ , ζ are shown (Figure 1A). The pattern of PKC α and PKC ζ is similar to previous reports and further confirms differentiation technique [17]. Our results establish that PKC β II levels rise concurrently with adipogenesis which represents a regulation of alternative splicing during differentiation but distinct from the rapid insulin-mediated switch (15 min) in PKC β splicing noted in skeletal muscle [4].

Effect of CGP53353 on 3T3-L1 adipocyte 2-[3 H]deoxyglucose uptake

We hypothesized that the increased levels of PKC β II were involved in some phase of glucose transporter translocation. We initially tried to reduce PKC β II using PKC β II specific siRNA and a variety of commercial transfection reagents. No significant knockdown was achieved (data not shown). Difficulty in transfecting differentiated 3T3-L1 adipocytes has been extensively published [18;19]. Prior success has been achieved in knocking down PKC β II in L6 skeletal cells in our laboratory [6]. To establish the involvement of PKCs other than atypical PKCs (aPKCs), we used LY379196 to assess its effect on ISGT since it only inhibits cPKCs and novel PKCs (nPKCs). Our results indicated that 25 and 50 μ M LY379196 inhibited ISGT (Figure 2A-B). To further determine the PKC isozyme specificity, we used CGP53353 which is a PKC β II specific inhibitor. To determine if CGP53353 could pharmacologically inhibit ISGT, we performed a dose curve response with

every well treated with insulin (Figure 2C). 50 μ M CGP53353 produced the greatest abrogation of ISGT. Next, we evaluated the effect of 50 μ M CGP53353 on 3T3-L1 adipocytes ISGT with controls for both CGP53353 and insulin treatment. Figure 2D shows ISGT decreased 85% with 50 μ M CGP53353. Basal glucose uptake was not altered by CGP53353. PKC β II protein expression levels are highest during the differentiated state whereas PKC β I and PKC α protein expression are low. Even though 50 μ M CGP53353 can inhibit other cPKCs, the effect on ISGT is due to specific inhibition of PKC β II [see discussion]. The specificity of CGP53353 was further examined and was found to be specific for suppression of phospho PKC β II Serine 660 (Ser660) [SF 2]. Taken together, these experiments indicated that PKC β II has a role in adipocyte ISGT.

PKC β II inhibition blocks insulin-stimulated GLUT4 translocation

Based on recent findings by Chappell et al. in L6 rat skeletal muscle cells, GLUT4 translocation was viewed as the most likely mechanism through which PKC β II was affecting adipocyte ISGT [6]. The intracellular pool of GLUT4 resides in the low-density microsomes (LDM) [15]. Upon insulin stimulation, GLUT4 translocates to the PM to facilitate ISGT. To determine if PKC β II was involved in insulin-stimulated GLUT4 translocation, we performed subcellular fractionation. 50 μ M CGP53353 inhibited insulin-stimulated GLUT4 translocation to the PM (Figure 3A-C). Comparing control vs. insulin treated cells, over 75% of LDM GLUT4 translocated, most of it presumably to the PM as seen by the increase in PM GLUT4 levels (Figure 3A,D). In contrast, PKC β II inhibition by CGP53353 prevented insulin from stimulating GLUT4 translocation from the LDM to the PM (Figure 3B,E). PM GLUT4 is 10 fold higher in insulin vs. CGP53353 with insulin treated cells indicating that inhibition of PKC β II blocks insulin-stimulated GLUT4 translocation (Figure 3A,D). In wells with CGP53353 alone, LDM showed slightly higher levels of GLUT4 compared to control LDM (Figure 3B,E). This could be attributed to PKC β II's potential role in GLUT4 priming or GLUT4 endosomal cycling. Cytoplasmic β -actin confirmed the loading of protein amount based on the BCA protein assay as well as confirming the results are not due to drug mediated cell death (Figure 3C). PM sheet assay was used as a second method to confirm a role for PKC β II in insulin-stimulated GLUT4 translocation [SF 3].

Akt phosphorylation is regulated by PKC β II

There are several mechanisms possible for PKC β II to regulate adipocyte glucose transporter translocation. One possibility is that PKC β II acts as a PDK2 or activates the PDK2 to phosphorylate Akt on Serine 473 (Ser473). PKC β II is a known regulator of Akt Ser473 phosphorylation in certain cell types [20]. To determine whether PKC β II had a role in phosphorylating Akt at Ser473, we treated 3T3-L1 adipocytes as shown in Figure 4 and performed western blot analysis. In agreement with the subcellular fractionation observations above, PKC β II inhibition blocked insulin-stimulated Akt phosphorylation at Ser473 by >94% (Figure 4A-B). This Akt phosphorylation site is known to be critical for insulin-stimulated GLUT4 translocation [21]. Akt phosphorylation at Thr308, a PDK1 site, was not significantly affected by PKC β II inhibition (Figure 4A-B). To confirm that Akt Ser473 was regulated by PKC β II, immunofluorescence was performed on 3T3-L1 adipocytes using treatments as above [SF 4].

CGP53353 has no significant effect on mTORC2 activity

Phosphorylation of Akt at Ser473 is dependent on mTORC2 activity. Both mTORC1 and mTORC2 share the mTOR protein which can be phosphorylated at several residues, including Thr2446, Ser2448 and Ser2481. Phosphorylation of mTOR at Ser2481 distinguishes activated mTORC2 from activated mTORC1 [22]. An initial co-immunoprecipitation experiment was performed to assess whether PKC β II could bind

activated mTORC2 in 3T3-L1 adipocytes. Figure 4C shows that there is an association between these two proteins mediated by insulin stimulation. Using whole cell lysates, CGP53353 inhibition of PKC β II had no effect on mTORC2 activity (Figure 4D-E). We believe this to be the first example conferring insulin-stimulated activation of mTORC2 in 3T3-L1 adipocytes. This is in agreement with results obtained in HEK293 cells treated with insulin [22].

Discussion

Here, we have shown that 3T3-L1 pre-adipocytes undergo developmentally regulated alternative splicing of the PKC β full length transcript during adipogenesis. Transcription and splicing factors responsible for this shift in isoform preference will need further verification but our results indicated that PU.1 and SRp40 binding to the promoter occurred in 3T3-L1 adipocytes (SF 1A-B) [23;24;25].

PKC β II was hypothesized to be critical in enabling GLUT4 to perform its physiological function of absorbing extracellular glucose in response to insulin, given its role in skeletal muscle ISGT [5;6]. Use of CGP53353 and LY379196 inhibitors has culminated in a narrow list of possible glucose uptake (and GLUT4 translocation) mediators. 25 μ M LY379196 was able to significantly inhibit ISGT thereby suggesting that there may be another PKC isoform involved other than the atypical PKC ζ (IC₅₀=48 μ M). LY379196 also helped to eliminate EGFR from possible CGP53353 effectors since LY379196 does not inhibit this protein [26]. EGFR is downregulated during 3T3-L1 differentiation and was not considered to be a factor in ISGT [27]. CGP53353 inhibits cPKCs, specifically PKC β II (IC₅₀=0.41 μ M), PKC β I (IC₅₀=3.8 μ M), PKC α (IC₅₀=1.9 μ M), PKC γ (IC₅₀=22 μ M) as well as EGFR (IC₅₀=0.7 μ M). Beyond 50 μ M, CGP53353 does not inhibit any other PKC isoform [28], including PKC ζ , the PKC isoform predominantly associated with 3T3-L1 ISGT [13]. PKC β I expression on day 8 and beyond, when glucose uptake assays were performed is virtually non-existent. It is therefore unlikely that it plays a significant role in glucose uptake. In addition, there is no documented role for PKC β I in insulin-stimulated glucose uptake in any cell line [5;29]. Next, Figure 2C shows that even at 20 μ M, CGP53353 is able to inhibit ISGT almost 45% (even though the intracellular concentration of CGP53353 is probably much lower due to the impermeable nature of differentiated 3T3-L1 adipocytes) PKC γ too is unlikely to be a major factor. Both drugs inhibit PKC α with IC₅₀s close to that of PKC β II and for this reason, PKC α could not be completely excluded. However, PKC α has been shown to negatively regulate insulin receptor tyrosine kinase activity by interacting with IRS1 [30]. Combined with the fact that during the course of 3T3-L1 adipogenesis PKC α expression is downregulated, it is unlikely that CGP53353 is mediating its effects on ISGT through PKC α . This leaves PKC β II as the best candidate. PKC β II's role in 3T3-L1 adipocytes had not been elucidated. PKC β II has been shown to be critical in ISGT in skeletal muscle. PKC β II C-terminal mutant overexpression or CGP53353 (1 μ M) administration were able to significantly inhibit ISGT in L6 skeletal muscle cells [5]. LY379196 (3×10^{-8} mol/l) was able to inhibit ISGT in 6 day-old cultured myotubes [31].

There are at least two discrete signaling pathways involved in insulin-regulated GLUT4 translocation in muscle and fat cells. The first involves PI3K and the second involves the proto-oncogene c-Cbl. The two targets of PI3K that have been identified are serine/threonine kinase Akt and PKC ζ . PI3K activates Akt by generating phosphoinositides in the inner leaflet of the PM. Akt docks to this through a pleckstrin homology domain bringing it in close proximity with phosphatidylinositol-dependent kinase 1 (PDK1) [32]. The mechanism of PKC ζ activation is not known although it may involve dissociation from 14-3-3 proteins among other things [3]. Recent reviews describe PI3K signaling diverging into two post-PDK pathways. One pathway diverges to an atypical PKC pathway shown to be crucial for

activation of glucose transport in both muscle and fat cells. The other is the Akt-dependent pathway [13]. CGP53353 blocked Akt phosphorylation on Ser473 in 3T3-L1 adipocytes suggesting that PKC β II is upstream of Akt. PKC β II mediated Akt phosphorylation on Ser473 occurs as a cell type and stimulus-specific event [20].

Akt activation requires phosphorylation at Thr308 in the activation loop and Ser473 in the hydrophobic motif. Phosphorylation of Ser473 results in the interaction between the hydrophobic motif and the N-terminal lobe leading to activation. Phosphorylation of Thr308 is accomplished by PDK1. The mTORC2 kinase complex has been shown to be indispensable for Ser473 phosphorylation [33]. Hunter et al. have recently reported that the mTORC2 complex can be distinguished from the mTORC1 complex by phosphorylation of mTOR at Ser2481 [22]. Since CGP53353 treatment has no effect on insulin-stimulated mTORC2 activation, we propose PKC β II as the upstream kinase that regulates Akt phosphorylation at Ser473. Phosphorylation by PKC β II fully activates Akt which is required for GLUT4 translocation. In order for PKC β II to become catalytically competent, it requires sequential phosphorylations at Thr500, Thr641 and Ser660. PDK1 is responsible for phosphorylation at Thr500. The two remaining phosphorylations require both mTORC2 and PKC β II's own intrinsic kinase activity [33;34]. The most plausible explanation for the obtained results is that PKC β II associates with mTORC2 for either complete activation of the mTORC2/PKC β II complex or to provide substrate specificity for mTORC2. It is also possible that PKC β II is the elusive phosphatidylinositol-dependent kinase 2 (PDK2) downstream of mTORC2 but upstream of Akt. To the best of our knowledge, this is the first study demonstrating that PKC β II also plays a role in 3T3-L1 adipocytes ISGT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. 3T3-L1 PKC β protein and mRNA expression during differentiation

(A) 3T3-L1 pre-adipocytes were differentiated from day 0 through day 12. Whole cell lysates (50 μ g protein) from each day were run on an SDS-PAGE gel and probed with the indicated antibodies via western blotting. Experiments were repeated three times. (B) Graphical representation shows PKC β II/actin, where * represents a statistically significant increase in PKC β II protein level as compared with day 0. (C) Graphical representation of PKC β I/actin, where * represents a statistically significant decrease in PKC β I protein level as compared with day 0. Unpaired t-test ($p < 0.05$) was performed using Prism5 (GraphPad Software, Inc., LaJolla, CA, USA). (D) 3T3-L1 pre-adipocytes were differentiated from day 0 through day 10. Total RNA was extracted and 2 μ g from each day was used for reverse transcription. Real-Time PCR analysis was performed as described in Materials and Methods. * represents a statistical significance in terms of fold change of PKC β II/actin mRNA compared to day 0 (percent basal) using unpaired t-test, $p < 0.05$. Experiment was repeated three times.

Figure 2. PKC β II inhibition attenuates adipocyte glucose uptake

Day 8 to day 12 3T3-L1 adipocytes were serum starved for 4 hrs, $\pm$ 30 min CGP53353 or $\pm$ 30 min LY379196, $\pm$ 15 min 100nM insulin. Glucose uptake assay was performed as described in Methods and Materials. (A) LY379196 dose curve (all wells treated with insulin) where 50 μ M is able to significantly (*) attenuate glucose uptake compared to insulin alone. (B) Glucose uptake was significantly (*) inhibited with 25 or 50 μ M LY379196 comparing insulin vs. LY379196 with insulin. (C) Dose curve shows the percentage decrease in glucose uptake when comparing insulin vs. CGP53353 with insulin. (D) Graphical representation of 50 μ M treatment where * represents a statistically significant decrease in glucose uptake comparing insulin vs. drug with insulin, using unpaired t-test, $p < 0.05$. Experiments were repeated three times and performed on day 10 and day 12 3T3-L1 adipocytes (when PKC α and PKC γ levels are lower) with no change in insulin effects (data included).

Figure 3. GLUT4 translocation is blocked by CGP53353 inhibition

Day 8 3T3-L1 adipocytes were serum starved for 4 hrs, $\pm$ 30 min 50 μ M CGP53353, $\pm$ 100nM insulin 15 min. Subcellular fractionation was performed as described. 5 μ g for each sample of each fraction (A-C) was loaded onto a SDS-PAGE gel and immunoblotted with indicated antibodies. Graphical representation of PM GLUT4 (D) and LDM GLUT4 (E), where * represents a statistically significant change comparing insulin vs. CGP53353 with insulin using an unpaired t-test, $p < 0.05$. GLUT4 doublet is likely a proteolytic byproduct. Experiments were repeated on three occasions with similar results.

Figure 4. Effect of PKCβII inhibition via CGP53353 on Akt phosphorylation and mTORC2 activity

Day 8 3T3-L1 adipocytes were serum starved for 4 hrs, ± 30 min 50μM CGP53353, ± 100nM insulin 15 min. (A) Whole cell lysates were run on an SDS-PAGE gel and probed with respective antibodies. (B) Graphical depiction of pAkt/total Akt, where black bars represent pAkt Serine 473 / total Akt and white bars represent pAkt Threonine 308 / total Akt. * represents a statistically significant inhibition of phosphorylation of Akt at Serine 473 comparing insulin vs CGP53353 with insulin using unpaired t-test, $p < 0.05$. Experiments were repeated three times. Day 8 3T3-L1 adipocytes were treated as in (A) but harvested in non-denaturing lysis buffer. (C) Lysates were subjected to co-immunoprecipitation as described and then run on a SDS PAGE gel and western blotted using indicated antibody (D) Lysates were run on a SDS PAGE gel and western blotted using indicated antibodies. (E) Graphical representation of two independent experiments.