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Involvement of PKCα in insulin-induced PKCδ expression: Importance of SP-1 and NFκB transcription factors

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Abstract

Protein kinase C delta (PKCδ) is a key molecule in insulin signaling essential for insulin-induced glucose transport in skeletal muscle. Recent studies in our laboratory have shown that insulin rapidly stimulates PKCδ activity and increases PKCδ protein and RNA levels, and that the SP-1 transcription factor is involved in insulin-induced transcription of the PKCδ gene. Activation of SP-1 involves serine phosphorylation and translocation to the nucleus. In this study we examined the possibility that PKCα might be involved in serine phosphorylation and activation of SP-1. We found that insulin rapidly phosphorylates and translocates SP-1. In the cytoplasm, SP-1 was constitutively associated with PKCα, and insulin stimulation caused these proteins to dissociate. In contrast, in the nucleus insulin induced an increase in association between PKCα and SP-1. PKCα inhibition blocked insulin-induced serine phosphorylation of SP-1 and its association with PKCα in the nucleus. Inhibition of PKCα also reduced the insulin-induced increase in PKCδ RNA and protein in the cytoplasmic and nuclear fractions. We also attempted to determine if another transcription factor might be involved in regulation of PKCδ expression. We earlier showed that insulin did not affect nuclear NFκB levels. Inhibition of NFκB, however, increased insulin-induced increase in PKCδ RNA and protein in the cytoplasmic and nuclear fractions. Surprisingly, this inhibition reduced the insulin-induced increase in cytoplasmic and nuclear PKCα RNA and protein. Inhibition of PKCδ reduced IκBα phosphorylation as well as NFκB activation. Thus, PKCα regulates insulin-induced PKCδ expression levels and this regulation involves activation of SP-1 and NFκB.

Keywords: Transcription factors; Insulin signaling; Gene transcription; PKCα; PKCδ; SP-1; NFκB

The binding of insulin to its receptor initiates a cascade of events leading to activation of a number of signaling proteins [1]. Others and we have reported that among the proteins activated by insulin stimulation of skeletal muscle and fat cells are certain PKC isoforms, in particular PKCs α, βII, δ, and ζ [2–7]. PKCs βII and ζ are activated via products of PI3 kinase activity [5]. While the pathway for insulin stimulation of PKCδ and α in skeletal muscle is not known, it is clearly independent of PI3 kinase activity and appears to involve participation of Src tyrosine kinase [8]. We have reported that PKCδ is a positive regulator of insulin receptor (IR) tyrosine phosphorylation and internalization in skeletal muscle [9]. Moreover, blockade of PKCδ reduces insulin-induced phosphorylation of IR and inhibits insulin-induced glucose uptake. Moreover, PKCα appears to be a physiological negative regulator of the insulin cascade and is constitutively associated with IRS-1 [10,11]. On activation by insulin, PKCα disassociates from IRS-1 and permits continuation of the insulin signal. In addition to its metabolic effects, insulin is known to regulate the transcription of specific genes including not only those involved in mediation of metabolic effects but also transcription factors, oncogenes, hormones, and cytoskeletal and membrane proteins [12].

Recently, we reported that insulin induced increase in PKCδ RNA and protein levels [13], and that these effects are mediated by the SP-1 transcription factor [22]. Insulin

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induces serine phosphorylation and translocation of SP-1 to the nucleus, and inhibition of SP-1 reduces the insulin induced increase in PKCδ RNA and protein levels. SP-1 contains consensus phosphorylation sites for a variety of kinases including MAP kinase (Erk 1 and Erk 2) calmodulin kinase, casein kinases, and protein kinases (PK) A and C. Interestingly, PKCα, which is also rapidly activated by insulin in L6 skeletal muscle, reportedly serine phosphorylates SP-1 when co-transfected with Erk2 [14,15].

Insulin rapidly activates both PKCα and PKCδ upstream in the insulin signaling cascade [9–11]. These proteins appear to play complementary roles in this cascade. Preliminary studies in our laboratory indicate that insulin may also upregulate PKCα RNA and protein levels. The various interactions involving PKCs α and δ raise the possibility that there may be elements common to regulation of expression. Studies in Baf3 and 32D cells have shown that PKCα may participate in the regulation of PKCδ RNA levels both at the level of transcription as well as by prevention of degradation of PKCδ mRNA [16]. In this study, we have examined the possibility that insulin activation of PKCα may be involved in the effect of insulin to regulate PKCδ expression levels in skeletal muscle through activation of SP-1 transcription factor.

Methods

Materials. Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed using antibodies purchased from Bio-Rad (Hercules, CA, USA) and other reagents from Sigma (St. Louis, MO, USA). The following antibodies were used: anti-PKCδ and anti-PKCα (Santa Cruz Biotechnology), anti p-IκBα (Santa Cruze), anti-skeletal muscle β-actin (Sigma Chemicals), anti-phosphoserine (Biomeda), Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (Bio-Rad), anti-SP-1 (Delta Biolabs), and anti-NFκB (Santa Cruz Biotechnology). Leupeptin, aprotinin, PMSF, DTT, orthovanadate, pepstatin, and NFκB inhibitor were purchased from Sigma Chemicals. Insulin (HumulinR-recombinant human insulin) was purchased from Lilly France SA (Fergersheim, France). Rottlerin was purchased from Cal-Biochem.

Cell culture. L6 cells were grown in α-modified Eagle's medium supplemented with 10% fetal calf serum for 4 days post-confluence, with media changed daily; cells were allowed to differentiate spontaneously or by changing the media to one supplemented with 2% fetal calf serum.

Cytoplasm extract. Dishes were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached with cell scraper in Ripa buffer (50 mM Tris–HCL, pH 7.4, 150 mM NaCl, 1 nM EDTA, 10 mM NaF, 1% Triton X-100, 0.1% SDS, and 1% Na deoxycholate) containing a cocktail of antiproteases and antiphosphatases (Sigma). After scraping, the preparation was centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant from this step was designated the cytoplasm fraction.

Nuclear extracts. Dishes were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached with cell scraper in PBS. Cells were transferred to Eppendorf tubes and centrifuged at 2*g* for 15 min at 4 °C. The pellet was resuspended in buffer (1 M Tris, pH 7.5, 2.5 M NaCl, 2 mM EDTA, and 1 mM EGTA) containing cocktails of protease and phosphatase inhibitors (Sigma). The suspension was then homogenized in a Dounce glass homogenizer (30 strokes) and centrifuged at 4*g* for 15 min at 4 °C. The pellet was resuspended again in buffer 1 (0.5 M sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM Tris, pH 8, and 1 mM DTT) and centrifuged again at 23,100*g* for 45 min at 4 °C. The pellet from this step was resuspended in buffer 2 (buffer 1 without EGTA, with 0.6 M KCl) and left in ice for 30 min. The suspension was then centrifuged at 75,500*g* for 60 min at 4 °C. The supernatant from this step was designated the nuclear fraction.

Immunoprecipitation. Specific antibody to SP-1 (dilution 1:100) was added to 400 μg protein from cytoplasm or nuclear extract and was rotated continuously for 60 min at 4 °C. After the samples were rotated for 60 min, 30 μl of A/G Sepharose was added and rotated overnight at 4 °C. The samples were then centrifuged at 2,000*g* for 10 min at 4 °C, and the pellet was washed three times with buffer 2 with centrifugation at 2000*g* for 2 min at 4 °C. To this, 25 μl of sample buffer (0.5 M Tris–HCl, pH 6.8, 10% SDS, 10% glycerol, 4% 2-β-mercaptoethanol, and 0.05% bromophenol blue) was added. The suspension was again centrifuged at 500*g* (4 °C for 10 min), boiled for 5 min, and then subjected to SDS–PAGE.

Western blot analysis. Western blots were performed as described [13]. Equal protein loading of Western blots was confirmed by immunoblotting for skeletal muscle β-actin.

RT-PCR. Total RNA was obtained using RNeasy mini kit (Qiagen) from control and insulin stimulated cells. Reverse transcription was performed on 1 μg total RNA using One Tube RT-PCR system (Roche) and 10 μM specific primers designed based on reported sequences for PKCδ and S12 control (ribosomal RNA—housekeeping gene). The RT reaction was amplified for 40 cycles (94 °C, 1 min; 50 °C, 35 min; 60 °C, 1 min; 72 °C, 1 min; and 70 °C, 10 min). Finally, 50% of the amplified products were resolved on a 1% agarose gel.

Primer sequences for PKCδ, PKCα, and S12:

PKCδ:
I AATCCCTTCCTCACCCATC
II TTCCTGTTACTCCCAGCCTC
PKCα:
I TGAACCCTCAGTGGAATGAGTCCT
II ATGGCTGCTTCCTGGTCTTCTGAAG
S12:
I GGAAGGCATTGCTGCTGG
II CCTCAATGACATCCTTGG

Results

PKCα is involved in insulin-induced SP-1 activation

We reported that insulin rapidly serine phosphorylates SP-1 and induces it to translocate to the nucleus. The identity of the serine kinase responsible for insulin-induced serine phosphorylation of SP-1 is not known. Among the several serine-threonine kinases have been shown to be able to serine phosphorylates SP-1 is PKCα. As a first step to examine the possibility that PKCα is involved in the insulin-induced increase in PKCδ levels through SP-1 activation, we examined possible physical association between PKCα and SP-1. In these studies, SP-1 immunoprecipitated from control and insulin-stimulated L6 muscle cells was subjected to SDS–PAGE and then immunoblotting with specific anti-PKCα antibodies. The results are shown in Fig. 1. In the cytosolic fraction (Fig. 1A), SP-1 is constitutively associated with PKCα and following insulin treatment PKCα is induced to disassociate from SP-1. In contrast, in the nuclear fraction (Fig. 1B) there was no detectable association between SP-1 and PKCα under basal conditions, and insulin stimulation induced association between these two proteins.

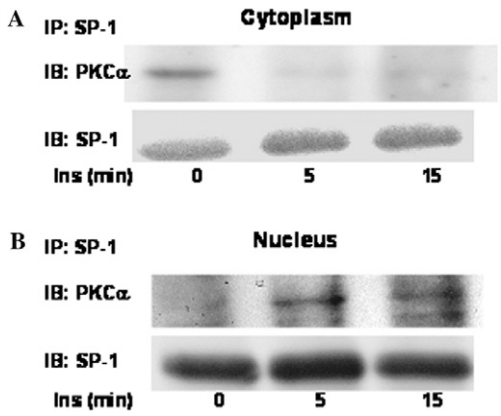

Fig. 1. Insulin induces disassociation between SP-1 and PKCα in the cytoplasm fraction and association between these proteins in the nuclear fraction. Differentiated myotubes were treated with insulin (10^{-7} M) for the times indicated, following which nuclear extracts or cytoplasm extract were prepared as described in the Methods. SP-1 was immunoprecipitated from the nuclear fractions and from the cytoplasm fraction with specific anti-SP-1 antibodies. The immunoprecipitated SP-1 was subjected to SDS–PAGE and immunoblotting with anti-PKCα antibody. (A) Western blots of insulin-induced de-association between SP-1 and PKCα in the cytoplasm fraction. (B) Western blots of insulin-induced association between SP-1 and PKCα in the nuclear fraction.

To further clarify the role of PKCα in SP-1 activation, we next examined effects of inhibition of PKCα on insulin-induced association between PKCα and SP-1 and serine phosphorylation of SP-1. PKCα was inhibited by treatment of muscle cells with Go6796, a selective PKCα inhibitor. Fig. 2 shows results obtained in these studies. Inhibition of PKCα prevented insulin-induced association between SP-1 and PKCα (Fig. 2A) and also reduced insulin-induced serine phosphorylation of nuclear SP-1 (Fig. 2B).

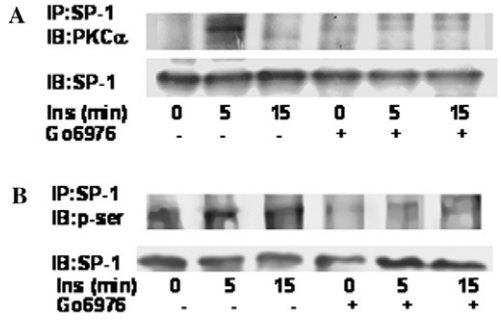

Fig. 2. Inhibition of PKCα reduces insulin-induced association between SP-1 and PKCα and SP-1 serine phosphorylation. Differentiated myotubes were pre-treated with PKCα inhibitor, Go6976 20 mM, for 30 min and then with insulin (10^{-7} M) for the times indicated. Nuclear extracts were prepared as in Fig. 1. SP-1 was immunoprecipitated from the nuclear fractions with specific anti-SP-1 antibodies. The immunoprecipitated SP-1 was subjected to SDS–PAGE and immunoblotting with anti-PKCα antibody and with anti-phosphoserine antibodies. (A) Western blots of Go6976 decrease insulin-induced association between SP-1 and PKCα in the nuclear fraction. (B) Western blots of Go6976 decrease insulin-induced serine phosphorylation of SP-1 serine phosphorylation of SP-1.

PKCα is involved in insulin-induced increase in PKCδ expression

We recently reported that SP-1 is essential for insulin-induced increase in PKCδ RNA and protein levels. Since, as we have shown here, PKCα is involved in SP-1 serine phosphorylation, we next considered the possibility that PKCα might participate in insulin-induced regulation of PKCδ RNA and protein levels. In these experiments we studied effects of Go6976 inhibition on insulin-induced increases in PKCδ RNA and protein levels. As can be seen in Fig. 3, inhibition of PKCα reduced insulin-induced increases in PKCδ protein levels in both the nuclear (Fig. 3A), and cytosolic (Fig. 3B) fractions as well as in PKCδ RNA levels (Fig. 3C).

NFκB involved in insulin-induced increase in PKCα

Because PKCδ plays a key role in many cellular processes as well as in the insulin signaling cascade it is reasonable to assume that that SP-1 may not act alone to regulate PKCδ gene expression. One candidate transcription factor might be NFκB, which was found to be involved in regulation PKCδ by TNF-α in keratinocytes. The NFκB binding

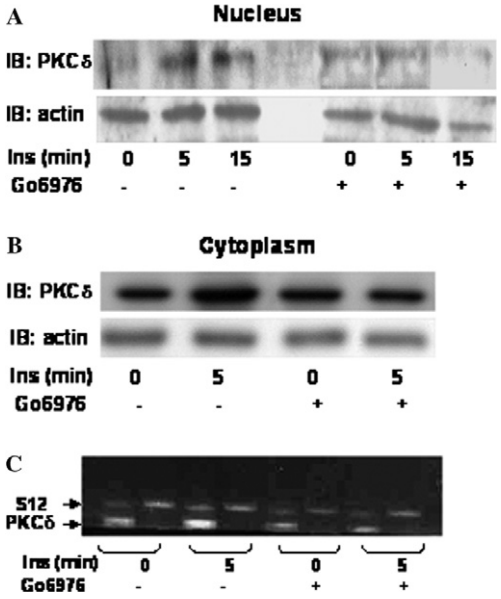

Fig. 3. Inhibition of PKCα reduces insulin-induced increase in PKCδ protein and RNA levels. Differentiated myotubes were pre-treated with PKCα inhibitor, Go6976 20 mM, for 30 min and then with insulin (10^{-7} M) for the times indicated, following which nuclear extracts or cytoplasm extracts were prepared as described in the Methods. Following SDS–PAGE and transfer, proteins were probed with specific antibodies against PKCδ. (A) Western blots showing that Go6976 decreases insulin-induced increase in PKCδ expression in the nuclear fraction. (B) Western blots showing that Go6976 decreases insulin-induced increase in PKCδ expression in the cytoplasm fraction. (C) Differentiated myotubes were pre-treated with PKCα inhibitor, Go6976 20 mM for 30 min and then with insulin (10^{-7} M) for the times indicated following which RNA was purified as described in Methods. PKCδ RNA and S12 ribosomal RNA were determined by RT-PCR.

site is located immediately upstream of the PKCδ transcription site. Accordingly, we considered the possibility that NFκB transcription factor may also be involved in insulin regulation of PKCδ gene expression.

In this series of experiments, we first examined the effect of insulin on NFκB expression in the nuclear fraction. We have already reported that insulin treatment does not appear to affect the expression levels of NFκB [22]. Here, we investigated the possibility that NFκB activity may nonetheless influence the levels of PKCδ and PKCα. We first examined effects of NFκB inhibition on insulin-induced changes in expression and protein levels of PKCs α and δ. We inhibited NFκB in two ways: (1) treatment of L6 muscle cells with NFκB inhibitor as well as by (2) expression of IκBα (kindly supplied by Prof. D. Wallach, Weizmann Institute of Science, Rehovot, Israel), which is unable to be phosphorylated and therefore remains bound to NFκB thus preventing its activation. The results of these studies are shown in Figs. 4 and 5. NFκB inhibition had opposite effects on insulin-induced changes in PKCα and PKCδ

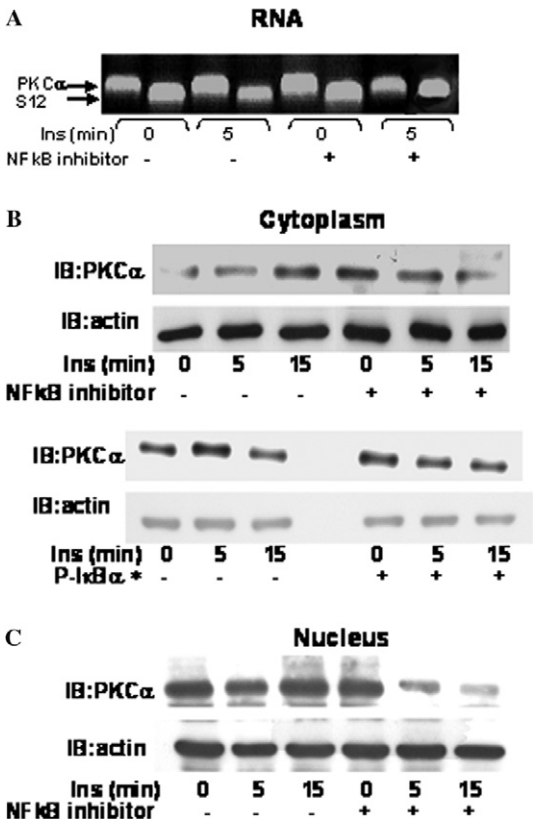

Fig. 4. NFκB activation inhibitor decreases insulin-induced increase in PKCα protein and RNA levels. Differentiated myotubes were pre-treated with NFκB activation inhibitor, [6-amino-4-(4-phenoxyphenylethylamino) quinazoline] 5 μg/ml for 12 h and then with insulin (10^{-7}M) for the times indicated. In addition, (B, right-hand blots), differentiated myotubes were transfected with mutant plasmid of IκBα, and then with insulin (10^{-7} M). (A) RNA was purified as described in Methods and PKCα RNA and S12 ribosomal RNA levels were determined by RT-PCR. (B) Cytoplasm extracts or (C) nuclear extracts were prepared as described in the Methods. Following SDS–PAGE and transfer, proteins were probed with specific antibodies against PKCα.

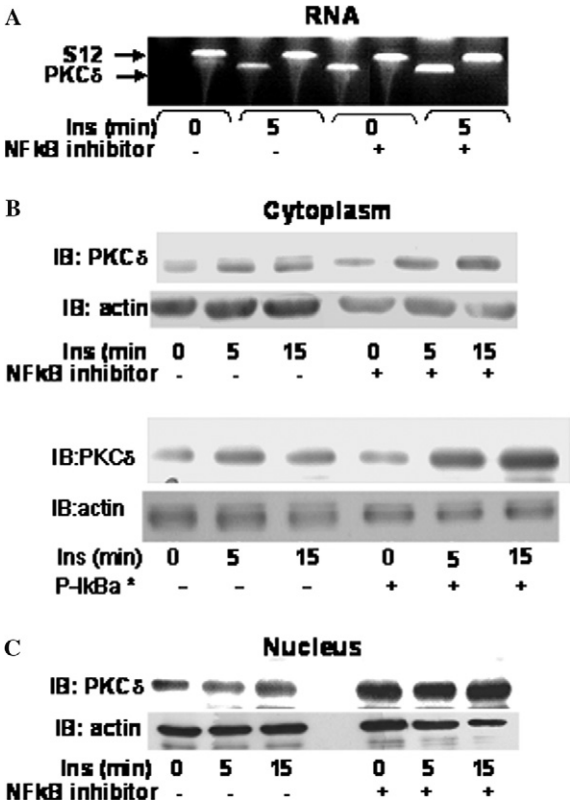

Fig. 5. NFκB activation inhibitor increases insulin-induced increase in PKCδ protein and RNA levels. Differentiated myotubes were treated as in Fig. 4. (A) RNA was purified as described in Methods, and PKCδ RNA and S12 ribosomal RNA levels were determined by RT-PCR. (B) Nuclear extracts or (C) cytoplasm extracts were prepared as described in the Methods. Following SDS–PAGE and transfer, proteins were probed with specific antibodies against PKCδ.

RNA and protein expression. As can be seen, inhibition of NFκB reduces insulin-induced increase in PKCα RNA (Fig. 4A) and protein both in the cytoplasm (Fig 4B) and in the nucleus (Fig. 4C). On the other hand, inhibition of NFκB increases PKCδ RNA levels (Fig 5A) and protein both in the cytoplasm (Fig. 5B) and in the nucleus (Fig. 5C).

PKCδ activity is necessary for NFκB activity

Studies have shown that PKCδ activation is necessary for NFκB activity [17,18]. Therefore, we next examined if inhibition of PKCδ may influence NFκB activation in skeletal muscle as well. Phosphorylation of IκB is necessary for dissociation of IκB from NFκB so that the latter can translocate to the nucleus where it can be activated. Inhibition of PKCδ was accomplished in two ways: (1) pretreatment of cells with Rottlerin (5 μM) for 1 h before addition of insulin, and (2) by infection of cells with adenovirus contstucts of kinase dead (dominant negative-DN) PKCδ (Kindly supplied by Prof. Chaya Brodie, Bar-Ilan University). The results demonstrate that inhibition of PKCδ by Rottlerin (Fig. 6A) or by DNPKCδ (Fig. 6B)

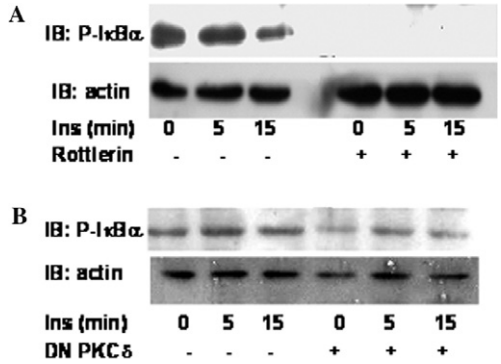

Fig. 6. Inhibition of PKCδ activation reduces phosphorylation of IκBα. Differentiated myotubes were treated Rottlerin or infected with adenovirus constructs of DNPKCδ following which they were stimulated with insulin (10^{-7}M) for the times indicated. Cytoplasm extracts were prepared and subjected to SDS–PAGE and transfer. Proteins were probed with specific antibodies against p-IκBα or β-actin to verify equal loading.

reduced phosphorylation of IKBα and consequently reduced NFκB activation.

Discussion

The results presented here clearly demonstrate that PKCα is involved in activation of SP-1 transcription factor. Inhibition of this isoform prevents insulin-induced physical association between SP-1 and PKCα, and reduces insulin-induced serine phosphorylation of SP-1. SP-1 transcription factor contains many phosphorylation sites that can be phosphorylated by serine threonine kinases. Interestingly, PKCα, which is also rapidly activated by insulin in L6 skeletal muscle, reportedly serine phosphorylates SP-1 when co-transfected with Erk2 [15]. Our findings show that PKCα is involved in insulin-induced SP-1 phosphorylation on serine residues as well. We also showed that both PKCα-dependent association of SP-1 with PKCα and SP-1 serine phosphorylation induced by insulin are essential for insulin-induced gene expression of PKCδ. The results reported here are consistent with our recent finding that the nuclear transcription factor SP-1 is involved in rapid insulin-induced transcription of PKCδ in skeletal muscle [22].

It has been reported that there is cross-talk between PKCα and PKCδ. Some studies show opposing roles for these two isoforms in the insulin signaling cascade. For example, PKCδ acts as positive regulator by physical association with IR [9] and promotes transmission of the insulin signal, while PKCα acts as a physiological negative regulator by association with IRS-1 and dissociates from IRS-1 in response to insulin-stimulation [10,11]. Other studies demonstrate relatively similar effects for PKCα and PKCδ. It was shown in several cell systems that overexpression of PKCα and PKCδ appears to exert the same biological effects. For example, overexpression of either PKCα or PKCδ causes prolonged activation of MAPK, induction of myeloid differentiation of murine 32D cells, and formation of polynuclear cells in hamster COH fibroblasts. It also was reported that there is a complex inter-isoenzyme relationship between PKCα and PKCδ in which activation, PKCα protein upregulates the steady-state levels of PKCδ mRNA, which causes an increase in PKCδ protein level [16].

PKCδ is a key molecule in many cellular processes and expressed at different levels in almost every type of cell. Hence, it would appear to be unlikely that SP-1 is the only transcription factor that involved in its regulation in skeletal muscle. The PKCδ promoter region has been sequenced and analyzed for putative transcription factor binding sites containing greater than 90% homology to the known consensus sequence motifs. It was found that the NFκB binding site is located immediately upstream of the PKCδ transcription site [19]. Furthermore, NFκB transcription factor was shown to be involved directly in TNF-α regulation of PKCδ expression in mouse keratinocytes. In a recent study [22], we investigated the involvement of NFκB in insulin-induced PKCδ and PKCα expression levels in skeletal muscle and did not find an effect of insulin on NFκB expression in the nuclear fraction. However, inhibition of this transcription factor induced an increase in insulin-induced increase in PKCδ RNA and protein levels both in the cytoplasm and in the nucleus, in contrast to effects of TNF-α on PKCδ expression in keratinocytes. On the other hand, inhibition of NFκB reduced the insulin-induced increase in PKCα RNA and protein levels both in the cytoplasm and in the nucleus. These results raise the possibility that NFκB may be involved in insulin-induced increase in PKCα which was found to be involved in insulin-induce increase in PKCδ gene expression via mediation of insulin-induced serine phosphorylation of SP-1 [22].

There are several studies that suggest a possible relation between regulation of expression of certain PKC isoforms and NFκB activation [17,18,20,21]. Thus, it was shown that phorbol esters, known activators of PKC proteins, induce activation of NFκB as well. In β cells, PKCβ phosphorylates IKKα and causes activation of NFκB. In PC12 cells overexpression of PKCγ is essential for activation of NFκB. Inhibition of PKCζ in *Xenopus* oocytes inhibits activation of NFκB. Finally, In airway epithelial cells activation of PKCδ increases association and transactivation of NFκB and in pancreatic acinar cells PKCδ and PKCε regulate activation of NFκB. When we inhibited PKCδ activation by Rottlerin or by adenoviruses with inactive kinase domain we observed a decrease in phosphorylation of IκBα. Our results indicate that activation of PKCδ is essential for phosphorylation of IκBα and, therefore, NFκB activation which depends on phosphorylation of IκBα.

In conclusion, the findings in this study show that PKCα regulates PKCδ RNA and proteins levels in skeletal muscle through activation of the transcription factor SP-1. PKCα expression levels appear to be regulated by NFκB whose activation depends on PKCδ activity. These regulations of PKCα and PKCδ, which are both rapidly activated by

insulin, indicate that there may be a reciprocal mechanism for regulation of these two proteins by insulin.

Acknowledgments

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