Protein kinase C (PKC) ε enhances the inhibitory effect of TNFα on insulin signaling in HEK293 cells

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Abstract Recently we have shown that PKC β1 and β2 are able to inhibit the tyrosine kinase activity of the human insulin receptor (HIR). Now we have investigated whether a distinct PKC isoform might be involved in the inhibitory effect of TNFα on insulin signaling in HEK293 cells. TNFα induces a rapid translocation of the PKC isoform ε (TNFα 10^{-9} M, maximal effect within 5–10 min) in rat-1 fibroblasts, while no effect occurred on other isoforms. Cotransfection of HIR with PKC ε did not significantly reduce the insulin stimulated receptor kinase activity; however, when cells were incubated with TNFα for 10 min (10^{-9} M) a 62 ± 17% ($n = 5$) inhibition of the insulin receptor kinase activity was observed which was significantly ($P < 0.01$) higher than that observed in cells which were not transfected with PKC (32 ± 11.5%, $n = 5$). The data suggest that translocation of PKC ε induced by TNFα enables this PKC isoform to interact with insulin signaling and to inhibit the insulin receptor kinase activity.

Key words: Tumor necrosis factor α; Insulin resistance; Protein kinase C; Insulin receptor; Insulin receptor substrate-1

1. Introduction

Protein kinase C (PKC) plays a key role in transmembrane signal transduction of several hormones, growth factors, and neurotransmitters. In recent years different isoenzymes have been identified and characterized according to their molecular and biochemical properties [1,2]. They represent a family of structurally and functionally related serine/threonine kinases which are derived from multiple genes as well as from alternative splicing of single mRNA transcripts [1,2]. The isoforms differ in their regulatory domains and dependence on Ca^{2+}, as well as in their tissue distribution. Due to those characteristics, PKC isoforms can be subdivided into three major groups: classical, Ca^{2+}-dependent cPKC isoforms (α, β1, β2, γ), new, Ca^{2+}-independent nPKC isoforms (δ, ε, η, θ), and atypical aPKC isoforms (ζ, λ). Activation of PKC seems to be associated with intracellular redistribution of the enzyme to the plasma membrane [1,2]. There is multiple evidence that PKC might be important as a modulator of insulin receptor function [3–5]. In addition we have shown that hyperglycemia induced insulin receptor inhibition seems to be mediated by PKC as well [6]. To investigate which PKC isoforms are inhibitory for the insulin receptor kinase activity we have recently studied the interaction of the insulin receptor with different PKC isoforms. HEK293 cells were transiently cotransfected with insulin receptor and the PKC isoforms α, β1, β2, γ, δ, ε, ζ and θ. After a prestimulation of the cells with phorbol esters we found that the PKC isoforms β1 and β2 were able to inhibit the insulin receptor kinase activity while other isoforms had no effect [7]. In another study in CHO cells an inhibitory effect on the insulin receptor activity could only be observed with PKC α [8]. Different mediators of insulin resistance have been discussed. Spiegelman et al. proposed a predominant role for TNFα in obesity induced insulin resistance based on studies in animal models, cell models as well as studies from human tissues [9–12]. In the present paper we have studied the impact of PKC on the TNFα induced insulin receptor kinase inhibition. Available data suggest that TNFα can activate PKC in certain cell lines [13–15]; however, there are at present no data concerning the question whether this activation of PKC is involved in the inhibition of insulin signaling. We have studied whether a specific isoform of PKC can be activated by TNFα and might be involved in the inhibition of insulin receptor kinase activity.

2. Material and methods

Cell culture reagents and fetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Human recombinant insulin was from Hoechst (Frankfurt, Germany). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), Na_3VO_4, Triton X-100, and dithiothreitol (DTT) were from Sigma (Munich, Germany). Reagents for SDS-PAGE and Western blotting were obtained from Roth (Karlsruhe, Germany) and Bio-Rad (Munich, Germany). Nitrocellulose was from Schleicher&Schuell (Dassel, Germany). Enhanced chemiluminescence reagents (ECL) were from Amersham (Braunschweig, Germany). Anti-pTyr antibody (PY20) was from Leinco (Baldwin, PA, USA). The insulin receptor antibody against the C-terminal domain of the β-subunit (αCT104) was described earlier [6]. Polyclonal PKC isoform ε antibody was purchased from Gibco (Eggenstein, Germany), and pan-PKC antibody was from UBI (Lake Placid, NY, USA), Qiagen Plasmid Kit was from Diagen (Hilden, Germany).

2.1. Transient expression of HIR and PKC isoforms in HEK 293 cells

The cDNA for the wild-type insulin receptor or different PKC isoforms (β1, β2, ε, and ζ) was cloned into a cytomegalovirus promoter based expression vector and plasmid DNA was prepared using a Qiagen Plasmid Kit. Human embryonic kidney (HEK) 293 cells (ATCC CRL 1573) were grown in Dulbecco's MEM/nutrient mix F12 medium supplemented with 10% fetal calf serum. A total of 4 μg plasmid DNA was transfected per semiconfluent 35 mm diameter dish according to the protocol of Chen and Okayama [16]. Cultures were maintained overnight at 37°C, 3% CO_2. Rat-1 fibroblasts were grown in 35 mm diameter dishes in DMEM-F12 medium supplemented with 10% fetal calf serum and 2 mM glutamine 20 h before

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Abbreviations: PKC, protein kinase C; HIR, human insulin receptor; IRS-1, insulin receptor substrate 1; TNFα, tumor necrosis factor α; CHO, Chinese hamster ovary; HEK, human embryonic kidney; DTT, dithiothreitol; FCS, fetal calf serum; ECL, enhanced chemiluminescence

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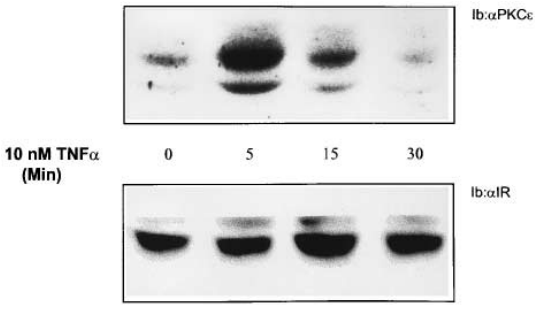

Fig. 1. TNFα induced translocation of PKC ε. Immunoblot showing the protein level of cPKC ε (upper panel) and the insulin receptor β subunit (lower panel) in the plasma membrane fraction of rat-1 fibroblasts overexpressing the human insulin receptor. Cells were incubated with 10^{-9} M TNFα for the times indicated. Plasma membrane fractions were separated by 7.5% SDS-PAGE and transferred to nitrocellulose. Nitrocelluloses were probed against a PKC ε specific or insulin receptor specific antibody as described. A representative immunoblot of three independent experiments is shown.

the experiment, the medium was changed to Dulbecco's MEM/nutrient mix F12 medium without FCS.

2.2. Stimulation of cells and separation of proteins

HEK293 cells and rat-1 fibroblasts were incubated with 10^{-9} M TNFα and 10^{-7} M insulin as indicated. Subsequently, cells were lysed in 200 μl lysis buffer (50 μM HEPES pH 7.2, 150 mM NaCl, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin).

For membrane preparation the same lysis buffer was used but without Triton X-100. The cells were centrifuged at 14000×*g* for 30 min and the pellet was solubilized with lysis buffer containing 1% Triton X-100. All lysates were centrifuged for 10 min at 14000×*g*, 40 μl of the supernatant was taken, 5×Laemmli buffer added, boiled for 5 min, and separated by 7.5% SDS-PAGE.

2.3. Western blotting

After electrophoresis, proteins were transferred to nitrocellulose by electroblotting (transfer buffer: 20 mM NaH_2PO_4 and 20 mM Na_2HPO_4, pH 8.8). After transfer, the nitrocelluloses were blocked with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100 and 0.25% gelatin, pH 7.4) for 1 h. Subsequently, they were incubated with the first antibodies (αPY20, αCT104 or PKC antibodies in NET buffer) overnight at 4°C. The nitrocellulose membranes were washed 4×10 min with NET buffer before incubating with horseradish peroxidase conjugated anti-rabbit IgG (for polyclonal antibody) or anti-mouse IgG (for monoclonal antibody) for 1 h at room temperature. Visualization of immune complexes was performed by ECL. To remove antibodies before reblotting the nitrocellulose was incubated in 62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol for 30 min at 55°C.

3. Results

TNFα induced PKC translocation was described earlier [13]. In this report the PKC isoform which responds to TNFα was not identified. In order to test the effect of TNFα on the subcellular distribution of PKC isoforms we prepared plasma membrane preparations from rat-1 fibroblasts overexpressing the human insulin receptor. To standardize the plasma membrane protein content we used the human insulin receptor as a marker protein. Fig. 1 shows that TNFα induced a rapid increase of the PKC isoform ε in the plasma membrane fraction suggesting a translocation of this PKC isoform. The lower panel showing the immunoblot of HIR demonstrates that equal amounts of plasma membrane protein are applied. TNFα had no effect on translocation of other PKC isoforms in rat-1 fibroblasts (data not shown).

In order to determine the impact of TNFα induced PKC ε translocation on the activity of the insulin receptor we cotransfected HEK293 cells with insulin receptor, IRS-1 and PKC ε. Control cells were transfected with HIR and IRS-1 alone or together with PKC β1, β2 or ζ. Fig. 2A shows insulin stimulated HIR and IRS-1 phosphorylation in these cells both in the absence and presence of 10^{-9} M TNFα. All cells have been stimulated with insulin (10^{-7} M) and an inhibitory effect

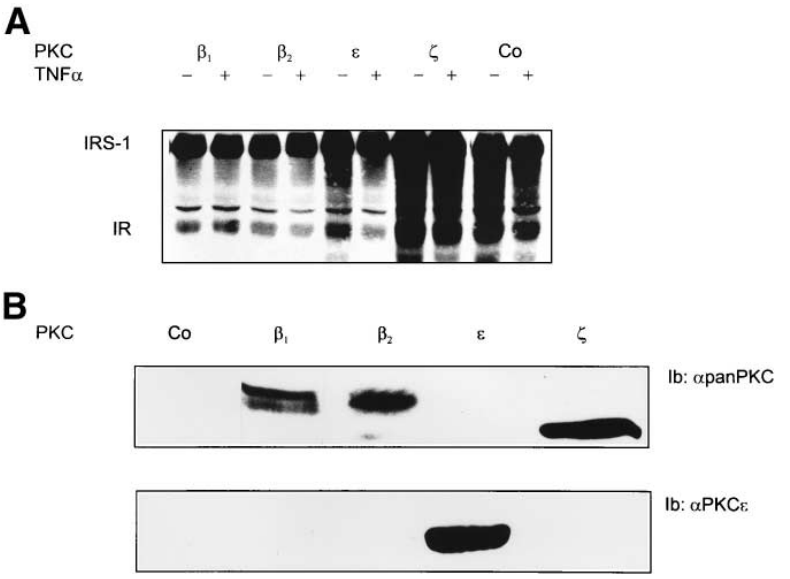

Fig. 2. Inhibition of insulin receptor autophosphorylation by TNFα. HEK293 cells were transfected with cDNA for the human insulin receptor, IRS-1 and different PKC isoforms as indicated. Transfected HEK293 cells were stimulated with insulin (10^{-7} M, 5 min) alone (−) or after preincubation (+) with 10^{-9} M TNFα for 10 min. Whole cell lysates were separated on a 7.5% SDS-PAGE, blotted onto nitrocellulose and probed against anti-phosphotyrosine antibody (A). Lysates from transfected cells were also probed with pan-PKC (crossreacts with β1, β2 and ζ PKC, but not ε PKC) and PKC ε antibody (B) indicating the expression levels of PKC isoenzymes.

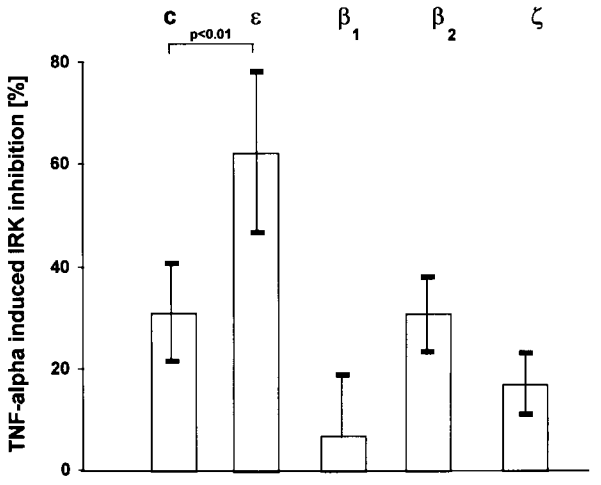

Fig. 3. Quantification of the insulin receptor kinase inhibition. Densitometric data are shown as mean ± S.E.M. of five independent experiments. Tyrosine phosphorylation of the insulin receptor in the presence of insulin (10^{-7} M, 5 min) was taken as 100% in all cells transfected with the different PKC isoforms. The effect of TNFα is expressed as percent inhibition of this value. Statistical analysis was performed by Student's *t*-test for paired samples.

of TNFα is seen in cells transfected with HIR and IRS-1 (Fig. 2A, Co). Earlier results were also confirmed by cotransfection of the insulin receptor with the PKC isoforms β1 or β2 which cause a decrease of the insulin stimulated receptor autophosphorylation [7]. In contrast, cotransfection of HIR and IRS-1 with PKC ε or PKC ζ showed no significant reduction of insulin induced receptor autophosphorylation compared to control cells which were transfected only with HIR and IRS-1 (Fig. 2A). A slight tendency to increased insulin receptor autophosphorylation was observed by cotransfection with PKC ζ (Fig. 2A). Different inhibition of HIR- and IRS-1 phosphorylation is seen after short term incubation with TNFα in cells cotransfected with PKC isoforms (Fig. 2A). While TNFα had only a small inhibitory effect on cells transfected with PKC β1, β2 and ζ, a clear inhibition was found in control cells, which was even more pronounced in cells transfected with PKC ε (Fig. 2A). Lysates were also blotted against anti-pan-PKC and anti-PKC ε antibodies which confirm overexpression of the corresponding PKC isoforms (Fig. 2B). Quantification of the data ($n = 5$) showed that only cotransfection with PKC ε can augment the inhibitory effect of TNFα (Fig. 3). These data suggest that PKC ε is able to interact in an inhibitory way with the insulin receptor/IRS-1 signaling chain after TNFα induced translocation.

4. Discussion

Studies in different cell systems have shown that TNFα inhibits insulin signaling at least partially by serine phosphorylation of IRS-1 [17–19]. It is at present unknown which serine kinase is involved in this effect. A role of PKC ζ, which is activated by TNFα stimulated ceramides, was discussed [14,15]. Beside this, ceramides are also able to activate protein phosphatases [20] which are also involved in the inhibitory effect of TNFα on insulin signaling as we have shown earlier [21]. However, a specific protein phosphatase has not been identified yet. We have observed a rapid inhibition of insulin receptor autophosphorylation and IRS-1 phosphorylation in rat-1 fibroblasts, NIH3T3 cells and HEK293 cells transfected with HIR and incubated with TNFα [21]. Since the unspecific PKC inhibitor H7 was not able to antagonize the inhibitory effect of TNFα we concluded that inhibition of insulin signaling by TNFα is unlikely to occur via activation of PKC. On the other hand there are reports demonstrating that TNFα is able to induce translocation of protein kinase C [13] and ceramide, a product of the TNFα stimulated sphingomyelinase has also been shown to activate PKC ζ [14,15]. These observations prompted us to readdress the question whether PKC dependent mechanisms might contribute to the crosstalk between the TNFα and insulin receptors.

The presented data suggest that in addition to the above described mechanisms a PKC ε dependent effect might indeed be important. This confirms the earlier observations of Krönke et al. [13] showing translocation of PKC by TNFα and demonstrates now an involvement of PKC ε for at least part of the inhibitory effect of TNFα. It is possible that TNFα induced PKC ε translocation enables this isoform to interact with the insulin receptor or IRS-1 probably in addition to other mechanisms. The insulin receptor kinase inhibition by TNFα is not enhanced by other PKC isoforms. In particular, we could not demonstrate reduced phosphorylation of the insulin receptor and IRS-1 after transfection with PKC ζ which can be activated by ceramides [14,15]. In contrast, cotransfection of PKC ζ with HIR and IRS-1 shows a slight increase of tyrosine phosphorylation in HEK293 cells. Furthermore, after cotransfection of PKC isoform β1 a less pronounced inhibition by TNFα compared to control cells was observed. This can be due to the fact that human insulin receptor activity is already suppressed by cotransfection with this PKC isoform. TNFα and PKC β might use same signaling ways for HIR inhibition which are already maximally activated by overexpression of PKC β1. In contrast PKC ε further enhances the inhibition of the insulin receptor autophosphorylation after TNFα stimulation in HEK293 cells. The mechanism of this increased TNFα effect is still unclear. A direct phosphorylation of IRS-1 on serine residues by PKC ε or activation of protein phosphatases seems possible and requires further studies.

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