PKC- α Modulates TGF- β Signaling and Impairs Podocyte Survival

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Key Words

PI3K • AKT • TGF- β

Abstract

Background: Progressive loss of podocytes has been documented as an early lesion in the development of glomerular disease. In a variety of glomerular diseases, including diabetic nephropathy the activation of transforming growth factor-beta (TGF- β) has been demonstrated to promote podocyte death and the development of glomerulosclerosis. In this manuscript we analyzed the role of PKC- α (PKC α) on TGF- β 1 induced apoptosis in podocytes. **Methods:** To accomplish this we generated stable murine PKC α deficient podocyte cell lines and examined survival- and pro-apoptotic signaling signatures as well as caspase activation after stimulation with TGF- β . **Results:** After stimulation with TGF- β we can demonstrate an enhanced and prolonged activation of PI3K/AKT and ERK1/2 in PKC α -knockout (PKC α -/-) podocytes compared to PKC α -wildtype (PKC α +/+) podocytes, whereas proapoptotic signaling via p38MAPK is significantly reduced. Interestingly, activation of the Smad-pathway is also prolonged in the PKC α -/-

podocytes. When we analyzed the underlying mechanisms we found a TGF- β inducible interaction of PKC α with the TGF- β -type-I-receptor (TGF β RI). Moreover, endocytosis assays showed that the TGF β RI is less internalized in PKC α -/- podocytes. **Conclusion:** Since we can demonstrate a key role for PKC α in the signaling response after stimulation with TGF- β we conclude that PKC α might be an interesting target molecule as a “podocyte protective” therapy.

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Introduction

Diabetic nephropathy is a severe complication of type 1 or type 2 diabetes and accounts for the most cases of end stage renal disease (ESRD) in the western countries [1]. The pathogenesis appears multifactorial in origin and involves various pathways such as TGF- β , advanced glycation end products (AGE), increased generation of reactive oxygen species (ROS) and activation of Protein Kinase C (PKC) [2]. Podocyte loss was noted in different genetic and toxic rodent models of diabetic nephropathy as well as in human disease [3-5]. Stimulation of podocytes with various components of the diabetic mi-

lieu induces proapoptotic signaling, production of ROS and caspase activation in podocytes [6]. However the influence and function of PKC on podocyte actions has so far not been studied systematically. The PKC family comprises 10 isoenzymes that are products of distinct genes and differ in their biochemical properties, tissue specific expression and intracellular localization [7]. PKCs are cytoplasmic serine/threonine kinases that are stimulated in response to growth factors, hormones or neurotransmitters [8]. The isoenzymes have been subclassified into three groups: The classical isoforms (PKC α , β I, β II and γ) require a lipid cofactor (phosphatidylserine (PS), Ca²⁺ and 1,2-diacylglycerol (DAG) for activation; the novel isoforms (PKC- δ , ϵ , η , θ , μ) are Ca²⁺ independent and the atypical isoforms (PKC ι / λ and ζ) require only PS [9]. In the kidney PKCs have been shown to be involved in the control of glomerular hemodynamics [10], tubular transport [11] and cell polarity [12]. With exception of the PKC ι / λ knockout, a PKC-isoform specific knockout does not regularly lead to a distinct phenotype. However in conditions with aberrant PKC-activation such as diabetes the role of different PKC-isoforms becomes apparent. In particular the α -isozyme (PKC α) has been shown to influence the renal outcome after diabetic injury. PKC α deficient mice show a better outcome after streptozotocin (STZ) induced diabetes with less glomerulosclerosis, less proteinuria, preserved nephrin expression and a higher number of WT-1 positive cells [13]. Since this could be interpreted as a podocyte protective effect, we wanted to examine the role of PKC α in podocytes and characterize the role of PKC α in central signaling pathways after TGF- β stimulation. Biochemical analysis revealed a central role for PKC α in TGF- β -Receptor endocytosis. This endocytosis mediation could account for the prolonged antiapoptotic signaling effect in PKC α deficient podocytes after TGF- β stimulation and thus explain protective cellular effects.

Materials and Methods

Antibodies and Cytokines

Primary antibodies that were used for Western blotting, immunohistochemical and immunofluorescence studies were rabbit anti-PKC α , rabbit anti TGFR1, rabbit anti β -Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-AKT, mouse anti-AKT, mouse anti-phospho-ERK, ERK, mouse anti-p38, rabbit anti-phosphop38, rabbit anti-Smad2, rabbit anti-phosphoSmad2 (Cell Signaling Technology Beverly, MA). Cytokines and inhibitors are purchased from

the following vendors: rhTGF- β (Cell Sciences, Canton, MA), PKC α -inhibitor (GÖ697) (Calbiochem, Merck, Darmstadt, Germany).

Animals

Targeted disruption and generation of PKC α -deficient homozygous mice on a 129/SV background is described elsewhere [13]. PKC α -deficient mice were crossed to immorto mice (BL6/C57 background) and used for generation of immortalized cell lines. The mice received a standard diet with free access to tap water. All procedures were carried out according to guidelines from the American Physiologic Society and were approved by local authorities.

Podocyte culture

Cultivation of conditionally immortalized mouse podocytes was performed as described by Mundel *et al.* 1996 [14]. To enhance expression of the thermosensitive T antigen cells were cultivated in the presence of 10U/ml IFN- γ (permissive conditions). For inducing differentiation, podocytes were maintained at 37°C for 14d without γ -IFN resulting in absence of thermosensitive T antigen (nonpermissive conditions).

Isolation of glomeruli and establishment of conditionally immortalized mouse podocyte cell lines

Glomeruli were isolated from kidneys of adult (9-week-old) mice using a sequential sieving technique with mesh sizes of 180, 100 and 71 μ m. The fraction collected from the 71 μ m sieve was maintained on collagen-I (BD Biosciences) coated flasks at 33°C. The medium consists of RPMI 1640 (Biochrom) containing 10% FCS (PAA), 1% penicillin-streptomycin (Gibco) and 10U/ml recombinant mouse γ -interferon (Cell Sciences). The outgrowing glomerular cells were cultured at 33°C in medium containing 100U/ml γ -Interferon. Limited dilution cultures of these primary cell lines resulted in selection of 10 monoclonal cell lines for each genotype. Only clones being positive for the podocyte markers Synaptopodin and WT-1 were further analyzed (at least 3 for each genotype). During 6 weeks of continuous culturing the γ -Interferon concentration was reduced to 10U/ml. Every experimental setup and result was confirmed in 3 different clones of PKC α ^{+/+} and PKC α ^{-/-} podocytes.

Western Blot Analysis

To analyse whole cell protein lysates from cultivated podocytes, either untreated or treated cells were lysed on ice in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % Nonidet P-40, 0.1 % SDS) containing protease inhibitor (Complete mini, Roche, Mannheim, Germany), 1 mM sodium orthovanadate, 50 mM NaF and 200 μ g/L okadaic acid. Lysates were centrifuged at 12,000 rpm and aliquots of the supernatants were separated by 10 % SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). After probing with primary antibodies antigen-antibody complexes were detected with horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies, respectively, and visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

Immunoprecipitation

For IP-Assay 500µg total cell lysate was incubated with 2 µg/ml IP-volume TGF-β-typeI receptor antibody and Sepharose-A beads in IP-buffer (25 mM Tris-HCL, pH 7.5, 1 mM DTT, 30 mM MgCl₂, 40 mM NaCL, 0.5% NP-40 and protease inhibitors) overnight at 4°C. The pellets were washed in IP-buffer and separated by SDS polyacrylamide gel electrophoresis. Western blot was performed using the methods mentioned above.

Transfection

The day before transfection the podocytes were seeded on coverslips (5000 cells/slide) and cultivated under permissive conditions. Cells were transfected using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol using mouse PKCα GFP3-tagged (kindly provided from Jae-Won Soh, Korea). After transfection cells were cultivated under nonpermissive conditions in normal growth medium for 72 h, serum-starved overnight and stimulated with PMA and TGF-β.

Endocytosis Assay

For internalization studies, cells were plated in 24-wells on glass slides and differentiated for 14d. Cells were incubated simultaneously with primary and Cy3 conjugated secondary antibody for 1h in RPMI without FCS at 4°C. After three washes with PBS cells were incubated for 20 min at 4°C or 37°C to induce internalization before fixing them with 4% paraformaldehyde.

Biotinylation of the TGFβ-R1

PKCα^{+/+} and PKCα^{-/-} podocytes were seeded on plates and differentiated for 14d. For Biotinylation cells were cooled at 4°C for 30 min to stop endocytosis. After that cells were incubated for 30 min at 37°C for endocytosis of the TGFβ-R1 or remained at 4°C as control. Cells were then washed three times with ice-cold PBS containing 1mM MgCl₂ and 0.1 mM CaCl₂ to remove any containing proteins. 80 µl of 10 mM Sulfo-NHS-SS-Biotin (Pierce) per milliliter PBS were added to the cells and incubated at room temperature for 30 min. After incubation cells were washed once with ice-cold PBS and non-reacted biotinylation reagent were quenched by incubation with 50mM Tris (pH 8.0) followed by three washes in ice-cold PBS. Cells were lysed in RIPA-buffer, centrifugated at 14.000 rpm for 15 min at 4°C and the resulting supernatant was incubated with 50 µl of 50% streptavidin agarose (Thermo Scientific) with rotating overnight at 4°C. After the beads were washed five times with RIPA-buffer, bound proteins were eluted with sample buffer by boiling for 5 min. Precipitated, biotinylated TGFβ-R1 was analyzed by immunoblotting.

Caspase-3/7-Assay

For detecting Caspase-3/7 Activity the Apo-ONE® Homogeneous Caspase-3/7 Kit (Promega) was used. Cells were seeded on 96-wells and differentiated for 14d. Cells then were stimulated with TGF-β (5 ng/ml) for 16 h. The assay was performed according to the manufacturer's protocol. Fluorescence was measured at 485_{Ex}/527_{Em}.

Fig. 1. TGF-β induces membrane translocation of PKCα in podocytes. (A) Characterization of generated podocytes. Western Blot demonstrates expression of PKCα and Nephtrin in generated immortalized murine PKCα^{+/+}, PKCα^{+/-} and PKCα^{-/-} podocytes. (B) PKCα^{+/+} podocytes were transiently transfected with a GFP-tagged PKCα expression construct or empty GFP-construct. Stimulation with PMA leads to a complete translocation of PKCα into the membrane. Stimulation with TGF-β (5 ng/ml) led to a partial translocation of PKCα after 5 min to the plasma membrane and induced perinuclear accumulation. (C) Effect of TGF-β on cytoplasmic and membrane levels of endogenous PKCα. Differentiated podocytes were stimulated with TGF-β (5 ng/ml) and fractionation of cytosol and membrane was performed as described in the materials and methods section. In untreated cells PKCα was predominantly in the cytoplasm (lane C). Treatment with TGF-β resulted in a translocation of endogenous PKCα from the cytoplasm in the membrane (lane M) after 5 min. Expression of PKCα in whole cell lysates of PKCα^{+/+} and PKCα^{-/-} is shown in lane L. The membrane specific protein Gli3 is only detectable in the membrane fraction and serves as control for purity of membrane fraction.

Results

TGF-β induces membrane translocation of PKCα in podocytes

To study the role of PKCα in podocytes we generated stable immortalized PKCα wildtype (PKCα^{+/+}) and PKCα deficient (PKCα^{+/-}, PKCα^{-/-}) podocyte cell lines

(Figure 1A). Each cell line was characterized for expression of podocyte specific proteins (see materials and methods section) and all results presented in the following were reproduced in three independent monoclonal cell lines per genotype. First we wanted to examine the activation kinetics of PKC α in podocytes. To accomplish this we transiently transfected podocytes with a PKC α -GFP construct (kindly provided from Jae-Won Soh, Korea) and stimulated the cells with 100nM PMA. We de-

tected a rapid membrane translocation of GFP-tagged PKC α within five minutes after stimulation (Figure 1B). Next we examined whether we can reproduce this rapid activation response of PKC α after stimulation with TGF- β . We were able to demonstrate that TGF- β can induce membrane and perinuclear translocation of PKC α in cultured podocytes in a similar time course as the classical PKC activator PMA (Figure 1B). When we stimulated cells transfected with an empty GFP-expression

Fig. 3. Inducible interaction of PKC α with the TGF- β R1. (A) TGF- β R1 was precipitated from whole cell lysates using anti-TGF- β R1 antibodies. The probes were blotted and analyzed for PKC α and TGF- β R1. (B, C) PKC α promotes TGF- β R1 endocytosis. (B) TGF- β R1 expressed at the surface of PKC α /+ and PKC α /− podocytes is labelled with an anti-TGF- β R1 antibody and Cy3-labelled secondary antibody. The cells are labelled at 4°C followed by an incubation at 37°C for 20 min to induce internalization in the absence or presence of the PKC α -Inhibitor GÖ6976 (20 μ M) (C). Western Blot demonstrates amount of biotinylated TGF β -R1 in PKC α /+ and PKC α /− podocytes before and after temperature shift for 30 minutes. TGF β -R1 expression was analyzed after surface biotinylation and precipitation with streptavidin-beads. Bar graph demonstrates merged densitometric data from two independent experiments. The results are shown as percentage of biotinylated receptor compared to control cells. Star indicates statistically significant difference of remaining, biotinylated TGF β -R1 in PKC α /+ cells compared to PKC α /− cells following temperature shift to 37°C (p-value (30 min) <0.01

vector plasmid we could not observe an accumulation of GFP after stimulation in a specific cellular compartment. Since we wanted to exclude that this translocation effect is influenced by artificial overexpression we performed membrane fractionation studies to detect membrane translocation of endogenous PKC α in podocytes after stimulation with TGF- β . Similarly to our findings with overexpression of GFP-PKC α we could detect translocation of endogenous PKC α to the membrane after stimulation with TGF- β in a similar time course (Figure 1C).

PKC α /−podocytes show enhanced activation profiles for PI3K-, ERK- and Smad-pathways and reduced p38MAPK-activation after treatment with TGF- β

First we wanted to examine the influence of PKC α on the PI3K/AKT and ERK-MAPK-pathways, since we had demonstrated in the past that the integrity of these pathways plays a central role for podocyte survival. Therefore we stimulated differentiated PKC α -wildtype (PKC α /+) and PKC α -knockout (PKC α /−) podocytes with TGF- β 1 (5 ng/ml) and harvested the cells at vari-

ous time points for up to 24 hours. When we performed westernblot analysis of AKT/PI3K and ERK1/2 pathways we found a significant enhancement of AKT- and ERK-phosphorylation in the PKC α /−podocytes for up to two hours (Figure 2A). In contrast, when we analyzed activation of p38MAPK pathways we found a significantly increased p38MAPK phosphorylation response in the PKC α /+ cells after TGF- β stimulation, whereas the phosphorylation response in the PKC α /− cells was significantly reduced (Figure 2B). These data indicate a severe shift in survival- and apoptosis-pathways in the PKC α /− cells. To test whether this shift in signaling pathways has functional consequences on the outcome after TGF- β 1, we performed caspase assays in PKC α /+ and PKC α /− podocytes. As expected we could demonstrate a significantly reduced caspase activation response in the PKC α /−cells after TGF- β 1 stimulation. (Figure 2C). Since we detected an enhanced AKT/PI3K and ERK-response in the PKC α /− podocytes after TGF β 1 stimulation, we wanted to analyze whether PKC α -deficiency would have an impact on Smad-signaling response as well. A modulation of Smad-dependent signalling by Protein

Fig. 4. Schematic overview of the role of PKC α in podocytes. Upon ligand binding the TGF- β receptor is phosphorylated and downstream activated PKC α leads to phosphorylation of proapoptotic p38MAPK. Activation of antiapoptotic PI3K/AKT and ERK1/2 is independent of PKC α activation, however PKC α promotes internalization and possibly recycling/degradation of TGF- β 1 receptor and thus influences signaling activity and activation profiles of PI3K/AKT and ERK1/2.

Kinase C has been observed in other cell types [15]. When we examined the Smad2-phosphorylation after TGF- β stimulation, we observed a significant enhancement in the TGF- β treated PKC α -/- podocytes (Figure 2D).

PKC α interacts with the TGF β R1 and promotes its endocytosis

Since the enhanced Smad2-phosphorylation in PKC α -/- podocytes implies a role for PKC α in the TGF- β pathway, we examined whether PKC α interacts with TGF- β R1 in podocytes. To demonstrate interaction we performed co-immunoprecipitation experiments in lysates of PKC α +/+ and PKC α -/- podocytes after stimulation with TGF- β 1. We could demonstrate an inducible interaction of PKC α with the TGF- β R1 in PKC α +/+ podocytes (Figure 3A). Since PKC α deficiency leads to enhanced and prolonged Smad2 phosphorylation in PKC α -/- compared to PKC α +/+ podocytes, we hypothesized that PKC α is involved in the turnover of the TGF- β R1. To test this, we performed endocytosis assays with the TGF- β R1 in PKC α +/+ podocytes, in the presence or absence of a PKC α inhibitor (GÖ6976) and PKC α -/- podocytes. Temperature shift from 4°C to 37°C induces internalization of the TGF- β R1 in endocytotic vesicles. This effect can be partially blocked in the presence of GÖ6976 and is completely absent in PKC α -/- podocytes (Figure 3B). To validate the enhanced endocytosis of the TGF- β -R1 in the presence of PKC α we performed biotinylation of the cell surface after endocytosis. After streptavidin-precipitation, the biotinylation assay indicated a significantly reduced

TGF β -R1-endocytosis in PKC α -/- compared to PKC α +/+ podocytes (Figure 3C). When we examined the expression of PKC α on tissue sections of patients with DN (n=4) we detected an upregulation of PKC α in podocytes in the biopsies from patients with DN compared to normal controls. However, broader studies revealed that this upregulation was not specific for DN as PKC α seemed upregulated in other diseases as well (data not shown). At this point it is tempting to speculate that PKC α activation could be a common mechanism in various glomerular diseases and more profound and extended studies are currently on the way in our laboratory to test this intriguing hypothesis.

Discussion

Our results provide novel mechanistic insights into the relationship of PKC α activity and the signaling response of podocytes. We demonstrate that PKC α is involved in endocytosis of the TGF β R1. Therefore we conclude that PKC α is a potential key regulator of podocyte survival and proteinuria development in glomerular diseases. Our conclusions are supported by the following lines of evidence:

Firstly, stimulation of differentiated podocytes with TGF- β leads to rapid accumulation of transiently overexpressed and endogenous PKC α in the plasma membrane and in the perinuclear compartment. The perinuclear translocation has been demonstrated to be essential in PKC α mediated activation of phospholipase D1 [16, 17]. The membrane translocation of

PKC α has been correlated with induction of apoptosis in other cell types [18, 19]. This translocation suggests activation of PKC α in podocytes after stimulation with TGF- β . Therefore one would expect a direct involvement of PKC α in signaling as well as in trafficking processes in podocytes.

Secondly, deficiency of PKC α leads to a significant change in pro- and anti-apoptotic signaling response in podocytes. PKC α -/- podocytes exhibit an enhancement of PI3K/AKT and ERK1/2 activity after TGF- β stimulation. We have shown previously that the integrity of the PI3K/AKT and ERK1/2 MAPKs response is essential for the survival of podocytes. Interferences in both pathways lead to a significant increase in podocyte apoptosis after stimulation with TGF- β [20-22]. Similarly, the caspase activation response is reduced in PKC α -/- cells after TGF- β stimulation. These findings are in line with observations in prostate cancer cells where PKC α mediates dephosphorylation and inactivation of AKT via activation of a PP2A phosphatase [23]. Similarly to the AKT response after stimulation we can document a higher amount of ERK-phosphorylation in the PKC α -deficient podocytes. This is in contrast to reports from other groups where ERK1/2 MAPKs activation was dependent on PKC α [24, 25]. However this seems dependent on the examined cell type, since in other cells PKC α inhibition led to increased ERK1/2 activity presumably via PKC dependent inhibition of MAPK-phosphatase expression [26, 27]. Another key mediator of proapoptotic responses as shown by us and others in podocytes is activation of p38MAPK. PKC α deficiency leads to a fundamental reduction of p38MAPK activation after TGF- β stimulation. We have demonstrated earlier that p38MAPK activation in podocytes is downstream of TGF- β [23]. Moreover, chemical inhibition of p38MAPK leads to a significant reduction of podocyte apoptosis [28]. PKC α has been implicated in the activation of p38MAPK in other cell types as well [29]. However it remains unclear whether this is a direct effect, or whether PKC α phosphorylates and activates kinases upstream from p38MAPK. These signaling effects lead to a significant reduction in the proapoptotic response in PKC α -/- cells after stimulation with TGF- β .

Taken together, we find a shift in pro-and antiapoptotic signaling response in PKC α -deficient cells. Interestingly, in this context we can demonstrate a dual role for PKC α in the MAPK activation- and deactivation-network. Pro-survival ERK1/2 MAPK pathways are less dephosphorylated in PKC α -/- podocytes, similar to the changed dephosphorylation response of AKT.

In contrast to that activation of pro-apoptotic p38MAPK is reduced (Figure 2).

Thirdly, we found that PKC α is involved in receptor endocytosis of the TGF- β receptor. The TGF- β pathway plays an important role in the diabetic milieu with very well documented direct effects on podocyte survival [30]. We found that TGF- β 1 induced phosphorylation of Smad2 is longer sustained in PKC α -/- podocytes. In addition we could document a TGF- β 1 inducible binding of endogenous PKC α to the TGF β R1. Moreover, functional PKC α is involved in endocytosis of the TGF β R1. Ranganathan and coworkers documented that PKC α mediated phosphorylation of a lipoprotein receptor orchestrates association with adaptor molecules and receptor endocytosis [31]. A similar function was documented for PKC α in fibroblasts for endocytosis of overexpressed Na $^{+}$ /H $^{+}$ exchanger isoform 3 (NHE3) [32]. On the basis of the published evidence and our results it is tempting to speculate that PKC α is a key mediator of receptor and membrane protein endocytosis in podocytes. We think that the continuance of active growth factor receptors on the cellular surface and the prolonged signaling answer in the absence of PKC α contributes to the changed signaling answer in PKC α -/- podocytes.

Based on our results we propose a new working model of PKC α functions in podocytes (Figure 4). In podocytes, PKC α changes the intracellular signaling response after stimulation with TGF- β . On the one hand PKC α dephosphorylates AKT and ERK1/2 MAPKs, on the other hand PKC α is a necessary cofactor for activation of p38MAPK. In the absence of PKC α the TGF- β R1 is less endocytosed. Therefore in podocytes deficient for PKC α the "positive" signaling effects are enhanced on various levels resulting in a beneficial net-signaling effect in response to TGF- β . So in summary PKC α inhibition is a promising target molecule for podocyte protective therapy in glomerular diseases.

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