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Suppression of protein kinase C-ζ attenuates vascular leakage via prevention of tight junction protein decrease in diabetic retinopathy

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ABSTRACT

To investigate the effect of protein kinase C (PKC)-ζ inhibition on vascular leakage in diabetic retinopathy, streptozotocin-induced diabetic mice were intravitreously injected with siPKC-ζ. According to the fluorescein angiography of the retinal vessels, suppression of PKC-ζ effectively attenuated vascular leakage in diabetic retina. Further evaluation on the retina with western blot analysis and immunohistochemistry revealed accompanying restoration of tight junction proteins on retinal vessels. As two major contributors to vascular leakage in diabetic retinopathy, vascular endothelial growth factor (VEGF) and advanced glycation end products (AGEs) were investigated on the tight junction protein expression in endothelial cells. Inhibition of PKC-ζ attenuated VEGF-induced decrease of tight junction proteins and accompanying hyperpermeability in human retinal microvascular endothelial cells (HRMECs). PKC-ζ inhibition also attenuated AGE-induced decrease of tight junction proteins in HRMECs. Our findings suggest that inhibition of PKC-ζ could be an alternative treatment option for compromised blood-retinal barrier in diabetic retinopathy.

1. Introduction

Diabetic retinopathy, the most common microvascular complication of diabetes [1], is the leading cause of blindness among working-aged adults [2]. The most frequent cause of the vision loss is diabetic macular edema [3]. The incidence of diabetic macular edema increased with more severe diabetic retinopathy, higher levels of glycosylated hemoglobin and longer duration of diabetes [4]. The severity of diabetic retinopathy was associated with levels of vascular endothelial growth factor (VEGF) in vitreous fluid [5]. As a potent inducer of vascular permeability, VEGF is known to play a critical role in diabetic macular edema [6]. Moreover, chronic hyperglycemia leads to the formation of glycosylated hemoglobin and advanced glycation end products (AGEs) [7]. The elevated serum levels of AGEs correlated with progression of diabetic retinopathy and macular edema [8,9]. Therefore, AGEs as well as VEGF may be primary contributors in the pathogenesis of diabetic macular edema.

The macular edema is intraretinal and subretinal accumulation of fluid caused by increased permeability of the perifoveal retinal capillaries [10]. The integrity of the blood-retinal barrier (BRB) is maintained by the presence of junctional molecules between adjacent endothelial cells and adhesive interactions between endothelial cells and neighboring pericytes [11]. As we have previously reported, zonula occludens (ZO) family is an essential component of tight junctions in retinal endothelial cells for maintaining BRB in diabetes [12–14]. Furthermore, expression of ZO family decreases earlier and more dramatically in highly permeable diabetic retina than that of occludin, whose phosphorylation affects endothelial permeability [12,15]. Therefore, decreased expression of ZO family likely contributes to increased permeability in diabetes.

Dysregulation of junctional molecules is mediated by a variety of mechanisms including the modulation of protein kinase C (PKC) activity [16]. PKC, a family of serine/threonine-related protein kinases, plays a key role in the induction of diabetic complications [17]. In diabetes, hyperglycemia leads to increased concentration of intracellular glucose, which then increases the synthesis of diacylglycerol (DAG), a main activating cofactor for PKC [17]. The PKC superfamily can be classified into three

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subfamilies: classical PKC (cPKC; α, β1, β2, and γ), novel PKC (nPKC; δ, ε, η, θ, and μ), and atypical PKC (aPKC: ζ, λ/ι) [18]. Activation of cPKC and nPKC is DAG dependent, while activation of aPKC is not DAG dependent [18]. DAG dependent subfamilies, cPKC and nPKC, were previously reported to be associated with vascular permeability in diabetes [12,19].

Although PKC-ζ is not yet proven to be involved in vascular permeability in diabetic retinopathy, PKC-ζ is likely to contribute to the BRB breakdown. PKC-ζ was detected in endothelial cells [20] and found to mediate the VEGF-induced proliferation and TNF-α and thrombin-induced hyperpermeability [21–23]. Furthermore, inhibition of PKC-ζ could prevent VEGF-induced tight junction breakdown and hyperpermeability in endothelial cells [24].

In this study, we demonstrated that suppression of PKC-ζ effectively attenuated vascular leakage in diabetic retina, which was accompanied by restoration of tight junction proteins on retinal vessels. Further *in vitro* study showed that inhibition of PKC-ζ attenuated VEGF- and AGE-induced decrease of tight junction proteins in human retinal microvascular endothelial cells (HRMECs). Therefore, our data suggest that PKC-ζ inhibition could prevent BRB breakdown in diabetic retinopathy by blockade of VEGF- and AGE-induced tight junction protein loss.

2. Material and methods

2.1. Mouse

Male C57BL/6 mice were purchased from Central Lab. Animal (Seoul, Korea). They were maintained on 12 h alternating light–dark cycles and served with food and water ad libitum. All animal experiments were approved by Institutional Animal Care and Use Committee of Seoul National University and conform to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Cell culture

HRMECs were purchased from the Applied Cell Biology Research Institute (Kirkland, WA, USA). HRMECs were cultured in M199 medium supplemented with 20% fetal bovine serum (FBS), 3 ng/mL basic fibroblast growth factor (Millipore, Billerica, MA, USA), 10 U/mL heparin (Sigma, St. Louis, MO, USA), and 1% (vol/vol) penicillin–streptomycin onto gelatin-coated flask. The cells were incubated in serum-free medium supplemented with VEGF (20 ng/mL, Cell Signaling Technology, Danvers, MA, USA) for 6 h or AGEs (10 ng/mL, Calbiochem, Billerica, MA, USA) for 24 h.

2.3. Induction of diabetes in mice

To induce diabetes, 10-week-old male mice were intraperitoneally injected with 180 mg/kg streptozotocin (Sigma). If plasma glucose concentrations were >300 mg/dL at 24 h after injection, mice were considered to be diabetic. To assess the effect of PKC-ζ inhibition, 1 μL of PKC-ζ inhibitor (pseudosubstrate peptide, 100 μg/mL; Calbiochem) or phosphate-buffered saline (PBS) was intravitreously injected to diabetic mice at 7 days after streptozotocin injection, and 1 μL of siPKC-ζ (20 μmol/L, Bioneer, Daejeon, Korea) or negative control siRNA (20 μmol/L, Bioneer) was intravitreously injected to diabetic mice at 6 days after streptozotocin injection.

2.4. siRNA Injection

It has been reported that siRNA can nonspecifically activate TLR3-mediated immune response in a length-dependent manner [25]. Thus, we used siRNAs with the same length, 21mer, to control for any length-related nonspecific action. PKC-ζ siRNA (Bioneer; 5′-CACAGAAACAGAACUCGAUTT-3′ and 5′-AUCGAGUUCUGUUUCUGUGTT-3′) and negative control siRNA (Bioneer; 5′-CUAUCAAGUGUCUCGUUGUTT-3′ and 5′-ACAACGAGACACUUGAUAGTT-3′) were used. The negative control siRNA was validated by vendor not to target any known genes in human, mouse and rat. The siRNAs were reconstituted according to the manufacturer's directions and injected 0.5 mm posterior to the limbus with a 33-gauge needle.

2.5. Assessment of retinal vascular leakage using fluorescein angiography

As we have previously described [13], at 8 days after streptozotocin injection, deeply anesthetized mice were perfused with 500-kDa fluorescein isothiocyanate–labeled dextran (FITC-dextran; Sigma) dissolved in PBS. The number of mice used in each experimental group was six. After 1 h perfusion, eyes were enucleated and fixed in 4% paraformaldehyde for 2 h. The retinas were dissected, flat-mounted in mounting medium (Dako, Glostrup, Denmark), and viewed by fluorescence microscopy (BX50, OLYMPUS, Tokyo, Japan) at 200× magnification.

2.6. Western blot analysis

Western blot analysis was performed using standard western blotting methods. Equal amounts of protein were separated by electrophoresis on 6–10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically onto nitrocellulose membrane (Amersham, Pittsburgh, PA, USA). After being blocked with skim milk, the membranes were incubated overnight at 4 °C with anti-ZO-1 (Invitrogen, Carlsbad, CA, USA), anti-ZO-2 (Invitrogen), and anti-phospho-PKC-ζ antibody (Thr410; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were visualized using a chemiluminescent substrate (Dogen, Seoul, Korea).

2.7. Immunostaining

The enucleated eyes were fixed in 4% paraformaldehyde and embedded in paraffin; 4 μm-thick serial sections were prepared, deparaffinized and hydrated. After being treated with proteinase K, slides were blocked with universal blocking solution (Biogenex, Fremont, CA, USA), incubated with anti-ZO-1 (1:100, Invitrogen) and anti-ZO-2 (1:100, Invitrogen) overnight at 4 °C. Negative controls were incubated without the primary antibody. After being washed, slides were incubated with biotinylated goat anti-rabbit antibody (Dako). Washed slides were incubated with Streptavidin-HRP conjugate (Vector Laboratories, Burlingame, CA, USA), developed using AEC substrate (Invitrogen). The expression of ZO-1 in cells was examined by an immunocytochemical method demonstrated previously [13]. Images were captured with a light microscope (Carl Zeiss, Chester, VA, USA) and a fluorescence microscope (BX50, OLYMPUS).

2.8. In vitro endothelial permeability assay

HRMECs (1×10^5 cells) were seeded onto type I collagen–coated Transwell unit (6.5 mm in diameter, 0.4 μm in pore size; Corning Costar, Cambridge, MA). After HRMECs became confluent, VEGF (20 ng/mL) was treated for 6 h. Then, 40-kDa FITC-dextran was added to the upper chamber at 100 μg/mL. After incubation for 30 min, the amount of FITC-dextran that diffused into the lower chamber was quantified by exciting at 485 nm and measuring the emission at 520 nm on spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.9. siRNA transfection

siRNA targeting PKC-ζ was chemically synthesized and purified in the 2′-deprotected and desalted form (Bioneer). The sequences of PKC-ζ siRNA pair were 5′-CACGGAAACAGAACUCGAU-3′ and 5′′-AUCGAGUUCUGUUUCCGUG-3′. Negative control siRNA (Bioneer) was used as a control for comparison. Transfection of siRNA was performed using lipofectamine and plus reagents (Invitrogen), according to the manufacturer's instructions. Briefly, HRMECs were seeded at 2×10^5 cells in a 6-well plate. After 24 h, cells were transfected at the final concentration of 20 nM siRNA. The efficacy of knockdown was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR).

2.10. RT-PCR analysis

Total RNA from cells and retinas was isolated using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. First-stranded cDNA was synthesized with 5 μg of total RNA and oligo(dT)18 primer by M-MLV reverse transcriptase (Invitrogen). Equal amounts of cDNA were subsequently amplified by PCR using PCR reaction kit (Bioneer) and specific primer for PKC-ζ and GAPDH. PCR products were separated on 1% agarose gels and visualized using SYBR® Safe DNA gel stain (Invitrogen) under UV transillumination.

2.11. Statistical analysis

All data are reported as mean ± SD. Student's *t*-test was used to evaluate which groups differed from the control group. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Suppression of PKC-ζ attenuates vascular leakage in diabetic retina

To investigate the effect of PKC-ζ suppression on vascular permeability in diabetic retina, siPKC-ζ or negative control siRNA was injected intravitreally at 6 days after streptozotocin injection. Retinal expression of PKC-ζ mRNA was evaluated at 8 days after streptozotocin injection. Intravitreal injection of siPKC-ζ suppressed retinal expression of PKC-ζ mRNA compared with eyes injected with same-length control siRNA (Fig. 1A). Whole-mount retina with intravitreal injection of siPKC-ζ was prepared after 1 h perfusion of FITC-dextran at 8 days after streptozotocin administration. As demonstrated in Fig. 1B, streptozotocin-induced diabetic retina showed markedly increased extravasation of FITC-dextran compared with the control. Suppression of PKC-ζ by siPKC-ζ dramatically prevented vascular leakage in diabetic retina while negative control siRNA did not show any anti-permeable effect.

3.2. Inhibition of PKC-ζ attenuates loss of tight junction proteins on retinal vessels in diabetic retina

Based on that increased vascular permeability of diabetic retina is associated with loss of tight junction proteins [12], the effect of PKC-ζ inhibition on tight junction proteins was evaluated in proteins extracted from diabetic retina. First, we observed decrease of ZO-1 and ZO-2 expression in diabetic retina compared with control. Then, we observed that the decrease was prevented by PKC-ζ inhibition with siPKC-ζ or PKC-ζ pseudosubstrate inhibitor (Fig. 2A and B). We previously demonstrated ZO-1 and ZO-2 significantly decreased even from 4 days after diabetes induction [12]. PKC-ζ inhibition at 6 or 7 days after streptozotocin injection effectively prevented further decrease of ZO-1 and ZO-2. As presence of junctional molecules between adjacent endothelial cells contributes to the BRB integrity [11], decreased expression of ZO-1 and ZO-2 on retinal vessels could be considered responsible for vascular leakage in diabetic retina. As we have expected, immunohistochemistry for ZO-1 and ZO-2 revealed their expression on retinal vessels in the control retina. The expression was markedly decreased in diabetic retina, whereas it was restored on retinal vessels by PKC-ζ suppression with siPKC-ζ (Fig. 2C). These results suggest that inhibition of PKC-ζ could attenuate vascular leakage in diabetic retina by modulating the expression of tight junction proteins on endothelial cells.

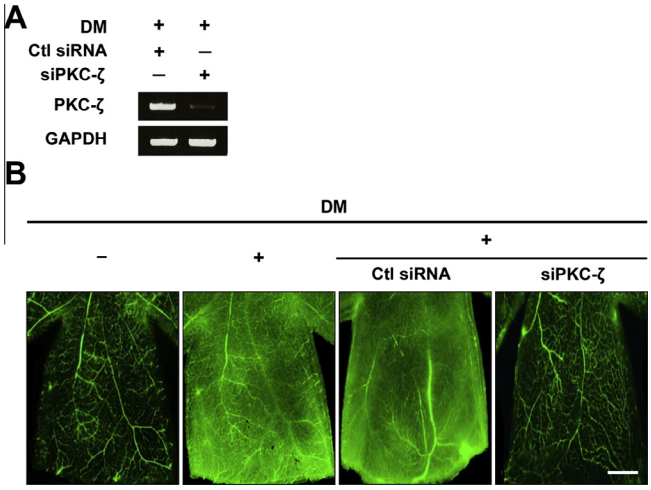

Fig. 1. Suppression of PKC-ζ attenuates vascular leakage in diabetic retina. (A) Retinal expression of PKC-ζ mRNA was evaluated by RT-PCR at 8 days after streptozotocin administration with intravitreal injection of negative control RNA (Ctl siRNA) or siPKC-ζ. RT-PCR was carried out using specific primers for PKC-ζ and GAPDH served as the loading control. (B) Vascular leakage in the retina was evaluated by fluorescein angiography using FITC-dextran. Eight days after streptozotocin administration with or without intravitreal injection of ctl siRNA or siPKC-ζ, whole-mount retina was prepared after 1 h perfusion of FITC-dextran. Figures were selected as representative data from three independent experiments. Negative control siRNA was used as a negative control. The scale bars represent 500 μm. DM, diabetes mellitus.

3.3. Inhibition of VEGF-induced PKC-ζ phosphorylation attenuates VEGF-induced decrease of tight junction proteins and accompanying hyperpermeability in HRMECs

We further investigated factors involved in the effect of PKC-ζ inhibition on the expression of tight junction proteins. Hyperglycemia in diabetes causes retinal capillaries to become incompetent functionally and anatomically inducing hypoxia in retinal tissues. This in return leads to release of VEGF that plays an important role in BRB breakdown in diabetic retinopathy [26]. In the streptozotocin-induced diabetic model, retinal VEGF levels were significantly higher compared with control [27]. Thus, the effect of PKC-ζ inhibition on VEGF-induced changes of tight junction proteins was investigated.

First, the effects of VEGF and PKC-ζ pseudosubstrate inhibitor on the activity of PKC-ζ were evaluated by western blot analysis of phosphorylated PKC-ζ expression. VEGF induced phosphorylation of PKC-ζ, and it was successfully inhibited by the PKC-ζ inhibitor (Fig. 3A). Then, the effect of PKC-ζ inhibition on tight junction proteins was evaluated. As shown in Fig. 3B, VEGF induced decrease of ZO-1 and ZO-2 and it was prevented by PKC-ζ pseudosubstrate inhibitor in HRMECs. The effect of restored tight junction

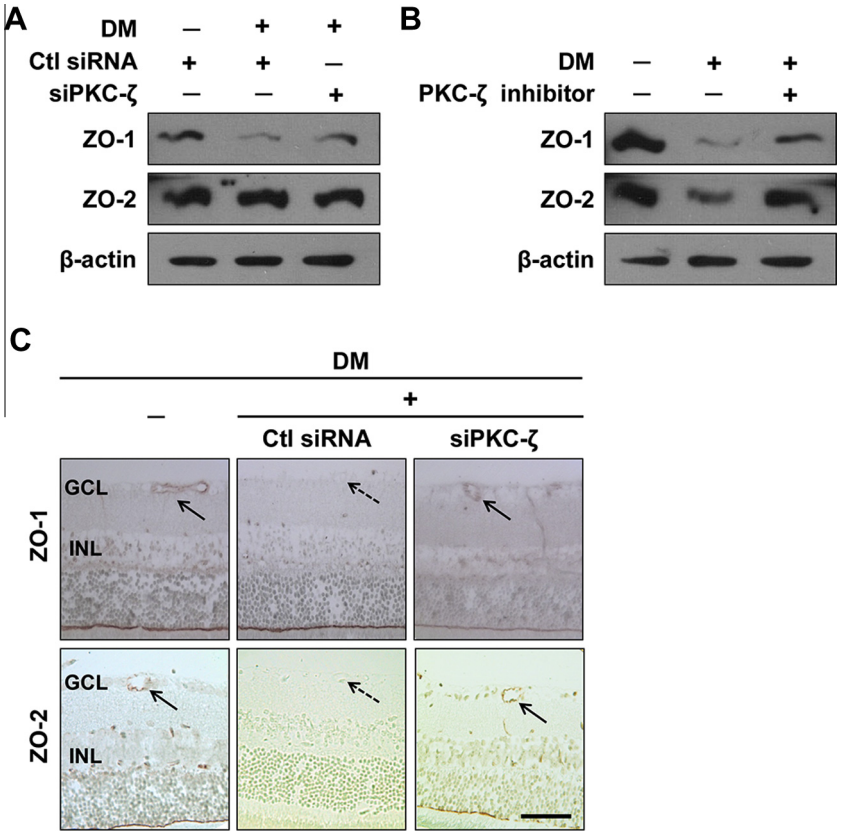

Fig. 2. Inhibition of PKC-ζ attenuates loss of tight junction proteins on retinal vessels in diabetic retina. (A and B) At 8 days after streptozotocin administration with or without intravitreal injection of PKC-ζ pseudosubstrate inhibitor, negative control siRNA or siPKC-ζ, retinal proteins were analyzed using antibodies to ZO-1 and ZO-2. β-actin served as the loading control. (C) Immunohistochemistry for ZO-1 and ZO-2 was performed in diabetic retina with or without intravitreal injection of siPKC-ζ. *Arrows* indicate ZO-1 (top) or ZO-2 (bottom) expression on retinal vessels, whereas *arrows with dotted line* indicate retinal vessels without expression of ZO-1 (top) or ZO-2 (bottom). Images were selected as representative data from three independent experiments. The scale bars represent 100 μm. GCL, ganglion cell layer; INL, inner nuclear layer.

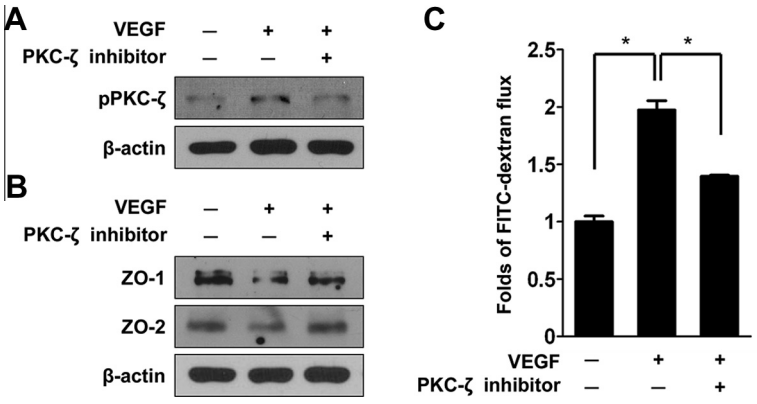

Fig. 3. Inhibition of VEGF-induced PKC-ζ phosphorylation attenuates VEGF-induced decrease of tight junction proteins and accompanying hyperpermeability in HRMECs. HRMECs were incubated for 6 h with VEGF (20 ng/mL) alone or in combination with PKC-ζ pseudosubstrate inhibitor (100 ng/mL). (A) Western blot analysis was performed to examine the expression of phosphorylated PKC-ζ. (B) ZO-1 and ZO-2 expression was assessed by western blot analysis. β-actin served as the loading control. Images were selected as representative of those in three independent experiments. (C) *In vitro* permeability was ascertained by quantifying the flux of 40-kDa FITC-dextran across cells grown on porous culture inserts. Each value represents the mean ± SD of results in three independent experiments (*$P < 0.05$).

proteins on VEGF-induced hyperpermeability was evaluated by quantifying the flux of FITC-dextran that passed through the endothelial monolayer. Six hours of treatment with VEGF induced hyperpermeability in HRMECs, which was inhibited by PKC-ζ pseudosubstrate inhibitor (Fig. 3C). Compared with the control, the FITC-dextran flux was increased to 1.97 ± 0.05 folds under the treatment of VEGF ($P < 0.05$). It was then effectively decreased to 1.39 ± 0.01 folds by additional treatment with the PKC-ζ inhibitor ($P < 0.05$). These results suggest that the effect of PKC-ζ inhibition on VEGF-induced loss of tight junction proteins could be responsible for its protective effect on vascular leakage in diabetic retinopathy.

3.4. Inhibition of PKC-ζ attenuates AGE-induced decrease of tight junction proteins in HRMECs

Another effect of hyperglycemia is formation of AGEs. AGEs are a heterogeneous group of highly glycosylated proteins or lipids

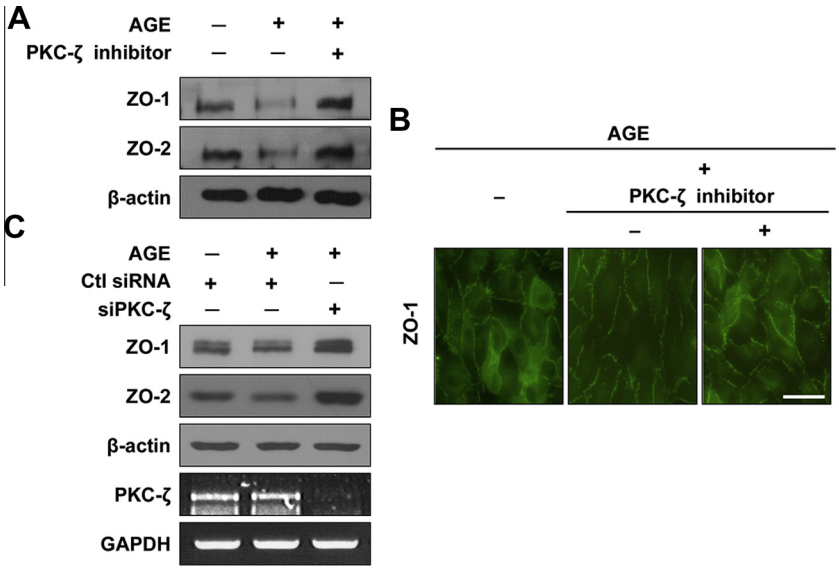

Fig. 4. Inhibition of PKC-ζ attenuates AGE-induced decrease of tight junction proteins in HRMECs. AGEs (10 ng/mL) were treated for 12 h with or without PKC-ζ pseudosubstrate inhibitor (100 ng/mL). (A) The expression of ZO-1 and ZO-2 was assessed by western blot analysis. (B) ZO-1 expression was evaluated by immunocytochemistry using antibodies to ZO-1. (C) HRMECs were transfected with negative control siRNA (Ctl siRNA) or siPKC-ζ. After 24 h, the transfectants were additionally incubated for 12 h in AGEs treatment. The expression of ZO-1 and ZO-2 was assessed by western blot analysis. RT-PCR was carried out using specific primers for PKC-ζ. β-actin and GAPDH served as the loading control. Images were selected as representative of those in three independent experiments. The scale bars represents 25 μm.

that form in hyperglycemic environments and contribute to the pathogenesis of diabetic retinopathy [28]. To investigate the effect of PKC-ζ inhibition on the expression of tight junction proteins in cells treated with AGEs, western blot analysis and immunocytochemistry of ZO family were performed after treatment with PKC-ζ pseudosubstrate inhibitor or transfection with siPKC-ζ. As shown in Fig. 4A, AGE-treated HRMECs showed decreased expression of ZO-1 and ZO-2, and PKC-ζ pseudosubstrate inhibitor effectively restored them. This result was confirmed by immunocytochemistry of ZO-1 in AGE-treated HRMECs (Fig. 4B). After assessing the efficacy of knockdown by siPKC-ζ with RT-PCR (Fig. 4C), HRMECs were transfected with siPKC-ζ. Suppression of PKC-ζ by siPKC-ζ also restored the expression of ZO-1 and ZO-2 (Fig. 4C). These results suggest that the effect of PKC-ζ inhibition on AGE-induced loss of tight junction proteins could be also responsible for its protective effect on vascular leakage in diabetic retinopathy.

4. Discussion

Previously, PKC-β and PKC-δ that belong to cPKC and nPKC respectively, were revealed to mediate BRB breakdown in diabetic retinopathy [12,19]. However, PKC-ζ that belongs to different superfamily, aPKC, has not been studied in the setting of diabetic retinopathy. Herein, we discovered, for the first time to our knowledge, that suppression of PKC-ζ could attenuate BRB breakdown in diabetic retinopathy.

This is very interesting finding because PKC-ζ seems to be different from cPKC or nPKC in regulating BRB integrity in diabetic condition. As a member of aPKC, PKC-ζ activation is not dependent on DAG that mediates activaton of cPKC and nPKC. Furthermore, retinal endothelial cells showed translocation of PKC-α,-β1,-β2, and PKC-δ but not PKC-ε or PKC-ζ in response to high glucose [29], which is another condition that can cause decreased expression of tight junction proteins [30]. We previously demonstrated translocation of PKC was responsible for the regulation of tight junction proteins [12]. As hyperglycemia and increased concentration of DAG are considered as important factors for the pathogenesis of diabetic complications [31], these unique features of PKC-ζ make it very unlikely for PKC-ζ suppression to be effective for maintaining BRB integrity in diabetic retinopathy. However, we demonstrated that PKC-ζ inhibition could prevent VEGF- and AGE-induced decrease of tight junction proteins, which was similarly reported in the effect of PKC-β or PKC-δ inhibition [12,32]. These *in vitro* results can explain unexpectedly effective *in vivo* data of PKC-ζ inhibition on reducing BRB breakdown in diabetic retinopathy.

However, PKC-ζ sometimes shows superior aspects in regulating tight junction proteins and endothelial permeability. Among PKC isoforms, PKC-ζ was the only isoform that was colocalized with ZO-1, which needed to be phosphorylated for proper assembly of tight junction [33,34]. Inhibition of cPKC or nPKC failed to prevent thrombin- and TNF-α-induced endothelial hyperpermeability that were effectively blocked by inhibition of PKC-ζ [22,35]. Further investigation is needed on the difference in mechanisms regulating tight junction proteins among PKC isoforms.

In this study, we demonstrated the vascular leakage in diabetic retina was effectively blocked by PKC-ζ suppression. This was accompanied by restoration of expression of tight junction proteins, and it was localized on retinal vessels where endothelial cells and junctional molecules are forming inner BRB. Further *in vitro* experiments were conducted on two major contributors of diabetic macular edema, VEGF and AGEs. In HRMECs treated with VEGF or AGEs, PKC-ζ inhibition successfully prevented tight junction protein decrease. Herein, we suggest that PKC-ζ inhibition could be an alternative therapeutic strategy to prevent BRB breakdown in diabetic retinopathy.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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