TLR4/PKCα/occludin signaling pathway may be related to blood-brain barrier damage

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Abstract. Abnormal blood-brain barrier (BBB) is a common pathological feature in brain damage. In the present study, a brain microvascular endothelial cell (BMEC) model was established to determine the role of the toll-like receptor 4 (TLR4)/protein kinase Cα (PKCα)/occludin signaling pathway in BBB dysfunction. Three small interfering (si)RNAs directed against PKCα were designed to investigate the molecular mechanisms of PKCα underlying BBB damage. BMECs were divided into 4 groups: Control group, TAK-242 (a TLR4 inhibitor) group, PKCα-siRNA group and TAK-242+PKCα-siRNA group. The results indicated that siRNA-3 was the most effective at silencing PKCα gene expression. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis indicated no significant difference of TLR4 mRNA expression levels between three different treated groups and the Control group. However, PKCα mRNA expression in the PKCα-siRNA and TAK-242+PKCα-siRNA groups were significantly decreased compared with that in Control and TAK-242 groups. In addition, occludin mRNA expression in PKCα-siRNA and TAK-242+PKCα-siRNA groups were significantly higher compared with the Control group. Meanwhile, occluding expressions in three treated groups were also significantly higher compared with the Control group. Furthermore, TAK-242 treatment, PKCα-siRNA treatment, and TAK-242+PKCα-siRNA treatment could promote occludin junctional labeling compared with the Control group. The permeability of PKCα-siRNA and TAK-242+PKCα-siRNA groups was significantly promoted compared with the control group. The TLR4/PKCα/occludin signaling pathway was closely related to BBB damage. The present study will lead to an improved molecular understanding of BBB damage in the future.

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Introduction

Deep hypothermic circulatory arrest (DHCA) has been widely used in cardiovascular surgery since its introduction by Griepp *et al* in 1975 (1). Despite its effectiveness in reducing mortality, prolonged DHCA (>40 min) inevitably leads to neuropsychopathic complications (2), such as temporary or permanent neurological dysfunction, which has a remarkable influence on prognosis. A number of experiments have revealed that disruption to the blood brain barrier (BBB) is probably a vital aspect in cerebral damage. The BBB is composed of endothelial cells and functions to create a 'protective bubble' around the brain. The BBB transendothelial electrical resistance induced by tight junction (TJ) impedes paracellular transport and maintains cell polarity (3,4).

TJ complexes comprise various transmembrane proteins, including occludin, claudins, junctional adhesion molecules and cytoplasmic plaque proteins, such as zona occludens protein (ZO-1, ZO-2 and ZO-3) (5,6). The first TJ protein identified in endothelial cells, occludin, interacts with ZO-1 in the cytoplasm. ZO-1 protein has shown to be closely related to α-catenin and the actin cytoskeleton (7). A previous study suggested that occludin-knockout treatments may significantly promote endothelial barrier permeability (8). Occludin-knockout mice exhibit normal TJ morphology and intestinal epithelial barrier function (9). Another study reported that low occludin expression levels had no influence on either the organization or the function of TJs (10). Therefore, it is speculated that TJ proteins are potential signaling molecules. Occludin and claudin proteins contain several phosphorylation sites, which have been indicated to increase protein internalization (11). Therefore, phosphorylation treatment may promote BBB leakiness. Notably, these phosphorylation sites are located in the consensus sequences of protein kinase

(PK)C and PKA (12), both of which are involved in occludin phosphorylation under normal and hypoxic conditions. PKC is a family of protein kinases that are involved in controlling the function of several other proteins through the phosphorylation of hydroxyl groups on the serine and threonine amino acid residues (13). PKC is activated by signals such as increased concentrations of dystroglycan (DAG) or cytosolic calcium ions (Ca^{2+}) (14). The PKC family comprises classical, novel and atypical isoforms. Classical PKCs, including PKCα, PKC-βI, PKC-βII and PKC-γ, require Ca^{2+}, DAG and a phospholipid, such as phosphatidyl serine, for activation (15,16).

TLRs are a family of receptors that monitor the presence of pathogens by their ability to identify microbial structural patterns. A previous study reported that bronchial epithelial cells express functional TLRs 1-6 and TLR9 (17). Atypical protein kinase C has been implicated in the regulation of the assembly of tight junctions in polarized epithelial cells (18) and previous studies have also identified signaling associations between TLR4 and PKC (19,20). For example, knockout treated TLR4 effectively decreased PKC-ζ expression and remitted liver damage in a study of acute pancreatitis (21). Meanwhile, PKC has also been shown to increase expression of lipopolysaccharides (LPS), which suggests that TLR4 activity is PKC-dependent (19). TLR4 is important in maintaining the enteric immune balance. Low expression of TLR4 and its downstream factor MyD88 exerts a protective effect on the whole immunologic balance (22). However, the overexpression of TLR4 is generally thought to serve a role in the activation of PKC and the induction of a series of inflammatory disorders (23,24).

Previous studies have reported that toll-like receptor 4 (TLR4) activates nonspecific PKC (19,21), which in turn accelerates the mobilization of TLR4 to lipid rafts. In addition, hypoxia-induced changes to the BBB involve increased paracellular permeability through a PKC activity-dependent mechanism, as demonstrated in both *in vitro* and *in vivo* conditions (25). Brain microvascular endothelial cells (BMEC), the major component of the blood-brain barrier, limit the passage of soluble and cellular substances from BBB. BMECs highly express genes associated with TJ molecules and efflux/influx transporters, and thereby could regulate the entrance of various types of compounds such as small molecules and drugs, into the brain. Therefore, BMECs have been proved to serve a critical role in the pathogenesis of many brain diseases (26). Based on the results mentioned above, we hypothesized that the TLR4/PKCα/occludin signaling pathway may be related with BMECs. Therefore, the relationships between PKCα and BMECs were investigated using a knockdown system. The mRNA expression and protein expression in different groups were tested. The results had identified our assumption that TLR4/PKCα/occludin signaling is closely related with BBB damage. This study may aid in advancing our understanding of the role of PKCα in the development of BBB damage.

Materials and methods

Cell cultures. All experiments were performed according to the relevant guidelines published by the Ministry of Agriculture of China. All protocols were approved by the Institute of Animal Sciences at the First Affiliated Hospital of Gannan Medical University (Ganzhou, China), where the experiments were conducted. Primary cultures of rat BMECs were collected from Wistar rats (aged 2 weeks) obtained from Sun Yat-sen University Experimental Animal Center (Guangzhou, China). Six male rats were housed in a pathogen-free facility with 12 h light/dark cycles and free access to food and water, and were acclimated for 1 week before experiments. The rats were sacrificed by cervical dislocation, and whole brains were quickly removed. After removing cerebellum, diencephalon (including hippocampus), white matter, residual vessels and cerebral pia mater, the cerebral cortex were washed with D-Hanks solution for three times. Then the cerebral cortex was cut into pieces by iris scissors and incubated with 0.1% II collagenase at 37˚C for 1.5 h. The supernatant was discarded, bovine serum albumin added and the sample centrifuged for 8 min at 4˚C (1,000 x g) to remove upper nervous tissue. It was then centrifuged again at 1,000 x g for 8 min at room temperature prior to being incubated with II collagenase for 1 h at 37˚C. Finally, 2 ml DMEM and 12 ml 50% Percoll were added and centrifuged at 1,000 x g for 10 min at 4˚C. The yellow layer was the purified BMECs. BMECs were maintained in a humidified chamber at 5% CO_2 and 37˚C.

According to the PKCα sequence from GeneBank (accession no. 002737), three small interference RNA sequences targeting PKCα were designed: siPKCα-1, 5'-GCGACATGAATGTTC ACAA-3'; siPKCα-2, 5'-GGAAGCAGCCATCTAACAA-3'; and siPKCα-3, 5'-GCTGGTCATCGCTAACATA-3'. These sequences were synthesized in Gene Pharma, Inc. (Shanghai, China) and used to build a PKCα-knockdown system according to previous protocol (27). The Control group comprised untreated BMECs. All lentiviral particles were generated by following a standardized protocol using the highly purified plasmids, EndoFectin-Lenti and TiterBoost reagents (Guangzhou FulenGen Co., Ltd., Guangzhou, China). The lentiviral transfer vector was co-transfected into cells with the Lenti-PacHIV packaging mix (Guangzhou FulenGen Co., Ltd.). The lentivirus-containing supernatant was harvested 48 h post-transfection and stored at -80˚C.

Cells ($5x10^5$ cells/well) were seeded in 6-well plates with Dulbecco's modified Eagle's medium containing 10% FBS (Thermo Fisher Scientific, Inc., Waltham, MA, USA) without penicillin and streptomycin overnight at 37˚C). Transfection was carried out with OPTI-MEM serum-free medium and Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), to achieve a final siRNA concentration of 100 nM (37˚C) (28). In addition, the TLR4 inhibitor TAK-242 (Takeda Pharmaceutical Company Ltd., Tokyo, Japan) was used to treat BMECs at concentrations recommended by a previous study (29).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The cells were seeded at a density of $1x10^6$ cells/well in 6-well plates. Total RNA was isolated from $1x10^6$ BMECs with the RNeasy Minikit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol with the DNase step. RNA (1 μg) was used for cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). mRNA transcript levels were quantify using the SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and

a 96-channel iCycler Optical system (Bio-Rad Laboratories, Inc.). The SYBR-Green-based reaction of 25 μl contained SYBR-Green Master Mix (1X), a gene-specific primer set (forward and reverse primers; 2.5 μM each), cDNA (20 ng), fluorescein calibration dye (10 nM; Bio-Rad Laboratories, Inc.) and ddH_2O. Each reaction was performed in triplicate. The primer sets for PKCα-siRNA-1, PKCα-siRNA-2, PKCα-siRNA-3, TLR4, occludin, and PKCα (mice species) were purchased from Genewiz, Inc. (Suzhou, China) and had the following sequences: PKCα-siRNA-1 forward, 5'-GCG ACATGAATGTTCACAA-3' and reverse, 5'-TTGTGAACA TTCATGTCGC-3'; PKCα-siRNA-2 forward, 5'-GGAAGC AGCCATCTAACAA-3' and reverse, 5'-TTGTTAGATGGC TGCTTCC-3'; PKCα-siRNA-3 forward, 5'-GCTGGTCAT CGCTAACATA-3' and reverse, 5'-TATGTTAGCGATGAC CAGC-3'; and β-actin forward, 5'-CAGGGCGTGATGGTG GGCA-3' and reverse, 5'-CAAACATCATCTGGGTCATCT TCTC-3' was used as the internal control. The cycling conditions were as follows: 1 cycle at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 45 sec. The iCycler-MyiQ software version 5.0 (Bio-Rad Laboratories, Inc.) was used to analyze the real-time fluorescence signals. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the β-actin gene (30).

Western blot analysis. Total cellular proteins were extracted from 1x10^6 BMECs under different treatments using phenylmethylsulfonyl fluoride (1%), radio immunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.1% SDS). Thirty μg proteins (24 g/μl) were boiled with SDS-PAGE sample buffer for 5 min at 100˚C and separated by 10% SDS-PAGE. The proteins were transferred onto a polyvinylidenedifluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% fat free milk powder for 1 h at room temperature, followed by incubation with the following primary antibodies: Rabbit polyclonal anti-GAPDH (cat. no. ab9485; 1:2,500; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-PKCα (cat. no. BA1355; 1:400; Boster Biological Technology, Wuhan, China), rabbit polyclonal anti-TLR4 H-80 (cat. no. sc-10741; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit polyclonal anti-occludin (cat. no. A2601; 1:1,000; Abgent, Inc., San Diego, CA, USA) at 4˚C overnight. The membranes were incubated with the paired secondary antibody [ProteinFind goat anti-rabbit IgG (H+L), HRP Conjugate, cat. no. HS101; 1:1,000; TransGen Biotech, Inc., Beijing, China] for 1 h at room temperature, and the separated proteins were detected with an Enhanced Chemiluminescence Detection kit (Advansta, Inc., Menlo Park, CA, USA). The bands were viewed with GeneGnome 5 (Synoptics, Ltd., Cambridge, UK) and ImageJ version 1.6.0 (National Institutes of Health, Bethesda, MD, USA) was used for densitometry.

Immunofluorescence staining. Immunofluorescence cell labeling was performed by rinsing 1x10^5 cells with cold PBS (pH 7.4; PAN-Biotech GmbH, Aidenbach, Germany), followed by fixing onto slides for 10 min in cold methanol at -20˚C, washing with PBS and incubating with the mouse anti-occludin primary antibody (cat. no. 33-1500; dilution, 2.5 μg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature.

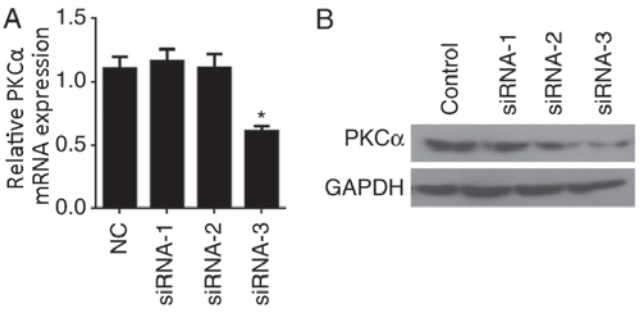

Figure 1. PKCα knockdown treatments with three different siRNAs in BMECs. (A) Reverse transcription-quantitative polymerase chain reaction analysis of the PKCα gene with three different siRNAs in BMECs. (B) Western blot analysis of PKCα protein with three different siRNAs in BMECs. siRNA-3 appeared to be the most effective at reducing PKCα mRNA and protein expression levels. All experiments were performed three times; *P<0.05 vs. untreated Control; BMEC, brain microvascular endothelial cell; PKC, protein kinase C; siRNA, small interfering RNA.

This was followed by an additional washing step with PBS, incubation with the Cy3-conjugated anti-mouse antibody (1:300; Jackson Laboratory, Ben Harbor, ME, USA) for 30 min at 37˚C, washing with PBS and mounted using Vectashield with DAPI (Vector Laboratories, Servion, Switzerland). Images were captured using a Zeiss 510 Meta confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany); 5 fields per slide were selected for calculating the intensity. ImageJ (version 1.6.0; National Institutes of Health) was used for intensity analysis.

Endothelial permeability assay. Fluorescein isothiocyanate (FITC)-labeled dextran (1 mg/ml; molecular mass, 40 kDa; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to measure the endothelial permeability through the BMEC monolayer. To measure paracellular permeability, around 3x10^5 BMECs were seeded on Transwell inserts (pore size, 0.4 μm; Corning, Cambridge, MA, USA) and cultivated for 2-4 days until a postconfluent cell monolayer was formed. EBM-2 medium with 0.2% FBS (CC-3156; Lonza, USA) was used in the upper and lower chambers. Following 30 min of treatment with 200 μg/ml FITC-coupled dextrans at room temperature, 100 μl culture medium was collected from the lower compartment and fluorescence was evaluated using a Paradigm Multi-Mode Plate Reader (Molecular Devices Shanghai Ltd., Shanghai, China), following the manufacturer's protocol.

Statistical analysis. Data are reported as the mean ± standard deviation. Student's t-test was used to compare the gene relative expression, relative fluorescence density and FITC intensity with different treatments. If more than two groups were compared, one-way analysis of variance was performed. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

PKCα-knockdown in BMECs. To knockdown PKCα gene expression in BMECs, three different siRNAs directed against PKCα, designated siRNA-1, siRNA-2 and siRNA-3,

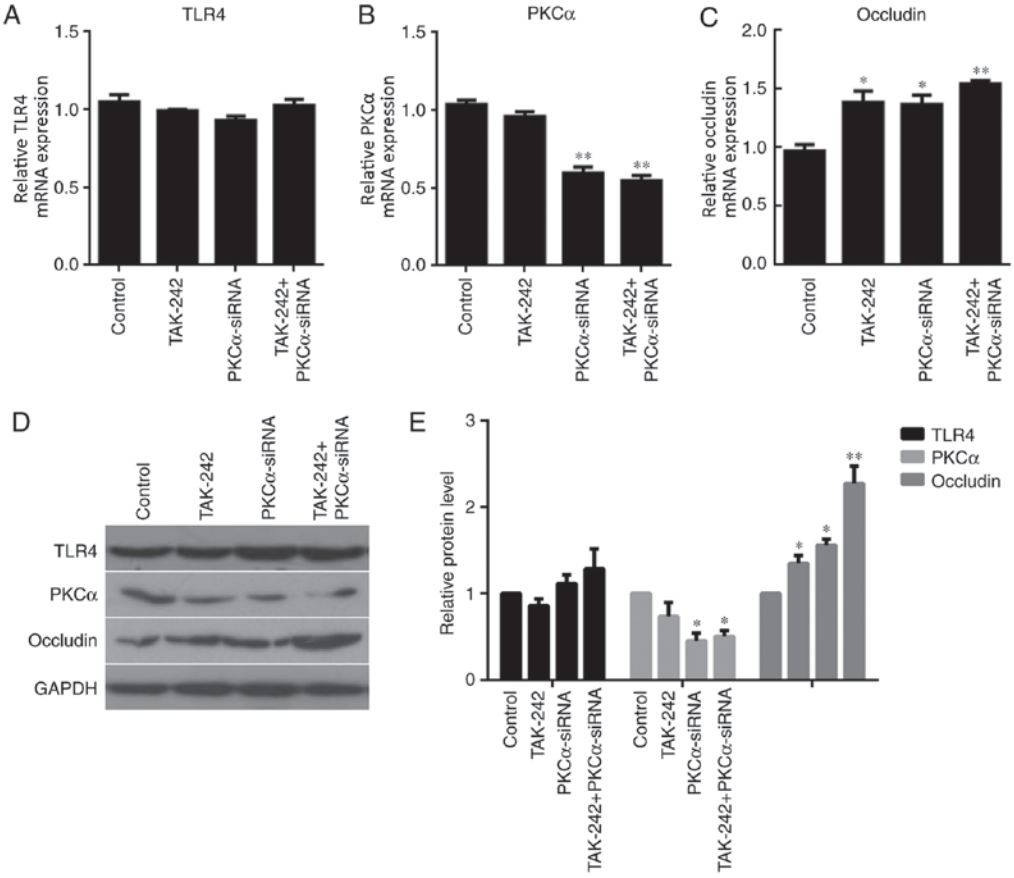

Figure 2. Effects on TLR4, PKCα and occludin expression in four different treatments in BMECs. BMECs were divided into four treatment groups and reverse transcription-quantitative polymerase chain reaction was used to examine the mRNA expression levels of (A) TLR4, in which no significant difference was identified between the groups; (B) PKCα, in which expression levels were significantly reduced in the PKCα-siRNA and TAK-242+PKCα-siRNA groups; (C) occludin, in which expression was significantly increased in all treatment groups. (D) Western blotting and (E) densitometric analysis of TLR4, PKCα and occludin protein expression levels in the four treatments of BMECs. *P<0.05 and **P<0.01 vs. Control. BMEC, brain microvascular endothelial cell; PKC, protein kinase C; siRNA, small interfering RNA; TLR, toll-like receptor.

were designed and to transfected into BMECs. The effects of siRNA transfection on PKCα mRNA and protein expression levels were examined by RT-qPCR and western blot analyses, respectively. RT-qPCR results indicated that siRNA-3 effectively reduced PKCα expression (P<0.05 vs. Control; Fig. 1A). Similarly, PKCα protein expression was notably reduced in cells transfected with siRNA-3 compared with expression in the Control, siRNA-1 and siRNA-2 groups (Fig. 1B). Therefore, siRNA-3 was used in all subsequent experiments.

TLR4, PKCα and occludin expression under different treatments. The mRNA and protein expression levels of TLR4, PKCα and occludin in BMECs were examined in the four different treatment groups. RT-qPCR analysis indicated no significant differences in TLR4 expression between the three different treatment groups compared with the untreated Control (Fig. 2A). However, PKCα mRNA expression levels were significantly reduced in the PKCα-siRNA treatment group and the TAK-242+PKCα-siRNA treatment group compared with expression levels in the Control and TAK-242-only groups (P<0.01; Fig. 2B). No significant differences were identified for PKCα expression between the Control and TAK-242 groups; no significant differences were identified for PKCα expression between the PKCα-siRNA and TAK-242+PKCα-siRNA groups. These results suggested that the alteration in PKCα expression was due to PKCα-siRNA treatment, not by TAK-242. The effects of the treatments on occludin mRNA expression levels were also examined. The results demonstrated that occludin mRNA expression was significantly increased in each of the treatment groups compared with the Control group (Fig. 2C). Nevertheless, there were no significant differences in occludin expression between the PKCα-siRNA and TAK-242+PKCα-siRNA groups. These data suggested that alteration in occludin expression may be triggered by PKCα-siRNA treatment. Meanwhile, TAK-242 treatment alone could also induce the increased occludin expression (Fig. 2C). No difference of TLR4 protein expression was observed between the 4 groups. PKCα protein expressions in PKCα-siRNA group (P<0.05) and TAK-242+PKCα-siRNA group (P<0.05) were significantly lower than that in Control group. Meanwhile, Occludin protein expressions in TAK-242 group (P<0.05), PKCα-siRNA group (P<0.05) and TAK-242+PKCα-siRNA group (P<0.01) were significant higher compared with the Control group. Western blot analysis revealed similar results as those of the RT-qPCR analysis (Fig. 2D and E).

Increased expression of occludin protein under different treatments. The expression and localization of the occludin protein

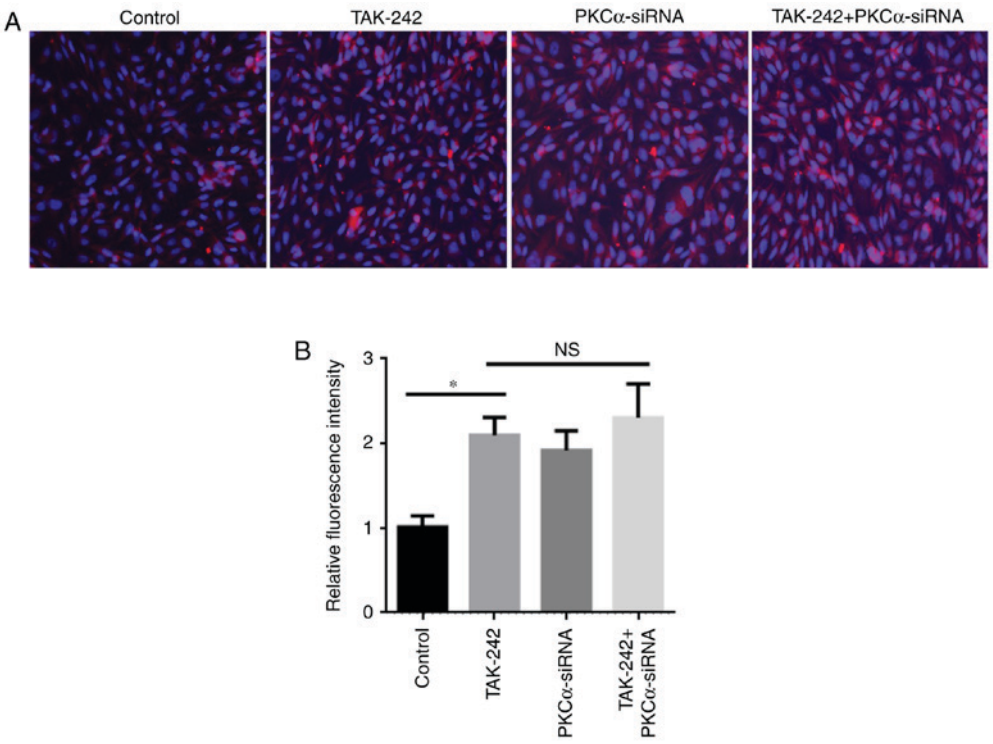

Figure 3. Immunofluorescence staining for occludin in BMEC cell monolayers in the four treatment groups. (A) Immunofluorescence staining with different treatments of BMEC. (B) Relative fluorescence intensity with different treatments of BMEC. There was an increased expression of occludin protein in all treatment groups. All experiments were performed three times; *P<0.05 vs. Control. BMEC, brain microvascular endothelial cell; NS, not significant; PKC, protein kinase C; siRNA, small interfering RNA; TLR, toll-like receptor.

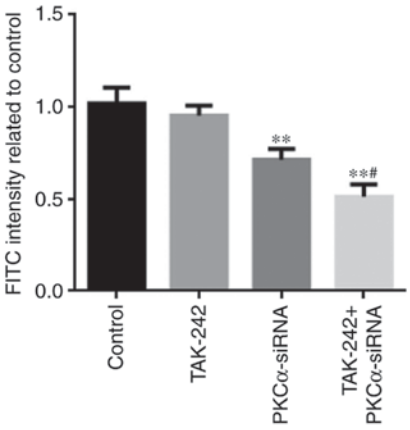

Figure 4. Alterations in endothelial permeability in BMECs in the four different treatments. Passage of FITC-labeled dextran through the BMEC monolayer was measured immediately following the four treatments. The results represent changes in permeability obtained in a representative experiment and are expressed as mean ± standard deviation in treated cells relative to untreated Control cells; n=3; **P<0.01 vs. Control; #P<0.05 vs. PKCα-siRNA.

were examined in the four BMEC treatment groups (Fig. 3). Immunostaining analysis of different BMEC treatments had demonstrated that occludin expression in TAK-242 group, PKCα-siRNA group, and TAK-242+PKCα-siRNA group were significantly increased compared with the Control group (Fig. 3B). Meanwhile, no significantly difference in occludin protein expression was observed between the TAK-242 and PKCα-siRNA groups. These results indicated that TAK-242 and PKCα-siRNA treatments may serve similar roles in the expression and location of occludin.

Permeability. Endothelial permeability was determined by measuring the passage of FITC-labeled dextran through the BMEC monolayer (Fig. 4). The permeability of PKCα-siRNA and TAK-242+PKCα-siRNA treated cells was significantly decreased compared with untreated Control cells (P<0.01). These results suggested that endothelial permeability decrease was mainly caused by PKCα-siRNA treatment, which could be promoted by adding TAK-242 to PKCα-siRNA treatment. However, it was notable that TAK-242 treatment alone could not induce any changes in endothelial permeability.

Discussion

As a gatekeeper, the BBB allows molecules that are required by the brain, such as oxygen and hormones, to pass into the brain extracellular fluid, and prevents other substances from entering, including metabolic and environmental toxins and bacteria. The BBB also actively transports essential molecules including TNF-α and interleukin in certain metabolic pathways. Clinical and experimental observations have indicated that hypoxia alters the integrity of the BBB; however, the intracellular signaling mechanisms that regulate TJ proteins at the BBB remain poorly understood (31). Although several studies have demonstrated the importance of PKC in regulating BBB function (32-34), the roles of PKCα, TLR4, and occludin in BBB regulation and their mechanisms are unclear. To determine the link between TLR4, occludin and PKCα activity, BMECs were used as a BBB model in the present study.

Occludin is considered to be the main sealing protein owing to its highly complicated structure (35), which comprises two equal extracellular loops, four transmembrane domains and

three cytoplasmic domains (36,37). The N-terminal site is crucial for its function and a mutant N-terminal may lead to the failure of complete TJ complex assembly (38). Vascular endothelial cells serve an important role in adjusting homeostasis and response to inflammation (39,40). Endothelial cells are regulated by physiological agonists and stress stimuli; endothelial cell activation is closely related with intracellular signaling pathways, including the synthesis and release of inflammation mediators, adhesion molecule induction and the actin cytoskeleton, which govern motility and permeability (41,42). Endothelial dysfunction may lead to damage to the endothelial integrity and cardiovascular pathology, such as atherosclerosis, hypertension and coagulation abnormalities (43). The crucial role of occludin to the integrity of tight junctions has been reported in both *in vitro* and *in vivo* studies (44,45). Knockout- occludin$^{-/-}$ treated mice showed morphologically intact tight junctions (46), but all showed poor TJ integrity and mucosal barrier dysfunction in further in-depth analyses. These data indicated an important role for occludin in TJ stability and barrier function. The present study evaluated the effects of four different treatments on the expression and localization of the occludin protein (Fig. 3). The results demonstrated that the expressions of occludin in the three treatment groups were significantly increased compared with the Control group. TAK-242 has been reported that it was an inhibitor for TLR4 (47). Therefore, it was hypothesized that TAK-242 treatment and PKCα-siRNA treatment should function through the same TLR4/PKCα/occludin signaling pathway, resulting in the similar occludin expression. The permeability observed in the PKCα-siRNA and TAK-242+PKCα-siRNA groups was significantly decreased compared with the Control group. The present study hypothesized that PKCα-siRNA treatment also was the major factor decreasing permeability, which was reinforced by TLR4 inhibitor treatment. However, TAK-242 treatment alone was not able to significantly decrease the BMEC permeability. RT-qPCR analysis indicated no significant difference of TLR4 expression in three different treatments compared with the Control group, which may be due to TAK-242 inhibiting TLR4 activity through protein level but not the mRNA expression level. However, this hypothesis requires further experimentation to verify. No significant difference was identified for PKCα mRNA expression and protein expression between cells treated with PKCα-siRNA and cells treated with TAK-242+PKCα-siRNA. This result indicated that the changes in TLR4 mRNA and protein should not be associated with PKCα expression. The expressions of occludin in TAK-242 group ($P<0.05$), PKCα-siRNA group ($P<0.05$) and TAK-242+PKCα-siRNA group ($P<0.01$) were significantly increased compared with the Control group. This implied that occludin expression could be changed by TAK-242 treatment, PKCα-siRNA treatment, or the two together. Western blot analysis was also performed to examine the effects of four different treatments on occludin protein expression, and this revealed similar results to RT-qPCR analysis.

The present study has a number of limitations, including the behaviors comparisons of different cell lines treated with PKCα-siRNA and a TAK-242 were not performed. The use of different cell lines may provide more comprehensive information about the TLR4/PKCα/occludin signaling pathway as it pertains to BBB damage. Another limitation is that animal experiments *in vivo* were not performed. The cell experiments may have been affected by multiple factors, such as cell state, environment and human factors. Animal experiments may effectively reflect the real regulatory process *in vivo*. Therefore, animal experiments are required.

In conclusion, PKCα may serve a negative feedback role in occludin expression, although no obvious connection was identified between PKCα and TLR4. However, PKCα-siRNA treatment was able to affect permeability, which was reinforced by the TLR4 inhibitor. However, TAK-242 treatment alone may not be effective in changing BMEC permeability. Understanding the specific roles of TLR4/PKCα and occludin in damage to the BBB may lead to an improved understanding of the molecular mechanisms in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and CX conceived and designed the study, and critically revised the manuscript. ZT and DG performed the experiments, analyzed the data and drafted the manuscript. LX, BW, XX, JF and LK participated in study design, study implementation and manuscript revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed according to the relevant guidelines published by the Ministry of Agriculture of China. All protocols were approved by the Institute of Animal Sciences at the First Affiliated Hospital of Gannan Medical University (Ganzhou, China), where the experiments were conducted.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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