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PKC and RhoA signals cross-talk in *Escherichia coli* endotoxin induced alterations in brain endothelial permeability

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ABSTRACT

Escherichia coli endotoxin LPS regulates blood–brain barrier permeability by disrupting the tight junction (TJ) complex between brain endothelial cells. This study used Bend.3 cells to examine the signaling networks involved in the hyperpermeability of the brain endothelial barrier caused by LPS. The LPS-induced alterations in the brain endothelial barrier were associated with PKC (a, β, ζ) and RhoA, but were independent of PI3 K and the tyrosine kinase pathway. Inhibition of PKC (a, β, ζ) and RhoA activity using shRNA and dominant negative mutants diminished the effects of LPS on the brain's endothelial TJs. The interactions between the PKC and Rho pathways were therefore examined. PKC-a and PKC-ζ, but not PKC-β interacted with RhoA in Bend.3 cells stimulated by LPS. PKC-a acted as the upstream molecule for Rho and PKC-ζ acted as the downstream target for Rho. Comparing the effect of double inhibition of "Rho and PKC" and single inhibition of "Rho" or "PKC" confirmed that this interaction is critical for LPS-induced brain endothelial cell hyperpermeability. Collectively these data are the first to suggest that LPS affects the brain's endothelial TJ barrier via PKC (a, β, ζ)- and RhoA, independent of the PI3 K and tyrosine kinase pathways. In addition, PKC-a and PKC-ζ, respectively, act as the upstream and downstream regulator for RhoA in the process.

1. Introduction

Despite the availability of effective antibacterial therapies, gram-negative bacterial meningitis in infants and children is associated with significant morbidity and mortality. Even survivors, 30–50% of them, sustain neurological sequelae [1]. Several lines of evidence indicate that the inflammatory response to bacterial lipopolysaccharides (LPS) promotes increased BBB permeability, leading to vasogenic brain edema as well as neuron death and dysfunction in severe cases [2]. Thus, LPS-mediated BBB dysfunction was thought to initiate or contribute to long-term neurological problems in patients with gram-negative bacterial meningitis.

Tight junctions (TJs) between brain microvascular endothelial cells (BMECs) form an important structural and functional basis to maintain BBB integrity [3]. Recent studies have shown that LPS, the major component of the outer membrane of gram-negative bacteria, could ruin the BMEC barrier by promoting TJ disassembly [4]. However, how TJs change in response to LPS is complicated and remains poorly understood.

In vivo and in vitro studies indicate that a complex network of signaling pathways is involved in regulating TJ structure and function, such as protein kinase C (PKC), tyrosine kinase, Ras or Rho GTP-binding proteins and phosphatidylinositol 3-kinase (PI3Ks) [5–8]. As PKC for example, with greater activity of PKC-α, the tight junctions lose their ability to form a tight barrier [9]. Nevertheless, it is interesting to note that various PKC subfamily inhibitors have confusing effects on TJ organization: some of them can block the TJ assembly and others may prevent TJ disassembly. The issue is which specific isoform is at play. The PKC family has 11 isoforms and can be divided into 3 classes: the conventional PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, μ and θ), and atypical PKCs (λ and ζ). Conventional PKCα and β as well as atypical PKCζ were thought to play important roles in promoting TJ dysfunction. while it is still uncertain how each PKC submits connects with TJ regulation in BMECs during LPS challenge.

RhoA is another key regulator of intercellular junction integrity. Pro-inflammatory mediators, including cytokines, thrombin and oxygen radicals, have been shown to activate the RhoA/Rho kinase pathway, inducing TJ disassembly and endothelial cell hyperpermeability [10]. Our previous study also showed that inhibition of RhoA markedly suppressed LPS-induced disruption of TJs [11].

Based on the above data, both Rho and PKC signals appear to participate in regulating BMEC barrier function under inflammatory conditions. Do they act simultaneously and independently or might one be a downstream target of the other? To further investigate the signaling pathways triggered by inflammation in the

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brain, the potential interactions between RhoA and PKC pathway should be elucidated.

Our experiments show that LPS-mediated TJ hyperpermeability is dependent on PKC (a, β, ζ) and RhoA activation, but is independent of tyrosine kinase and the PI3K pathway. In the above process, PKC-a and PKC-ζ signaling interact with RhoA signaling, while PKC-β and RhoA work independently.

2. Materials and methods

2.1. Reagents

Cell culture supplies were obtained from Invitrogen. LPS (*Escherichia coli* 055:B5), Gö6976, calphostin, wortmannin and PP2 were obtained from Sigma Chemical. C3 transferase was purchased from Cell Signaling. RhoA activity assay (pull-down) kit was purchased from Cytoskeleton. PKC activity assay kit was obtained from Immune Chem Pharmaceuticals Inc. Anti-ZO-1, anti-claudin-5 bodies were purchased from Santa Cruz Biotechnology. Anti-PKC-a, anti-PKC-β and anti-PKC-ζ bodies were obtained from BD Biosciences.

2.2. In vitro study

2.2.1. Cell culture

Bend.3 cells was kindly afforded by Dr. Jian Zhang (The University of Chicago, U.S.), were cultured in fibronectin-coated culture dishes or on fibronectin-coated cell inserts with 0.4 μm pore size.

2.2.2. Plasmids and transfection

PcDNA3.1hygro-N19RhoA plasmid, the dominant negative mutant of RhoA was synthesized at Minghong CO (CHN). An expression vector containing PcDNA3.1hygro plasmid alone served as the control of the PcDNA3.1hygro-N19RhoA plasmid.

PLKO.1-puro-PKCa-shRNA, PLKO.1-puro-PKCβ-shRNA, PLKO.1-puro-PKCζ -shRNA and empty PLKO.1-puro vector were kindly afforded by Dr. Jian Zhang (The University of Chicago, U.S.).

All of the plasmids were introduced into Bend.3 cells by using Lipofectamine 2000 according to the manufacturer's instructions. The stable transfected Bend.3/N19RhoA (N19RhoA) and Bend.3/vector-1 cells (empty PcDNA3.1hygro) were obtained using the Hygromycin B (400 ug/ml, Sigma) selection method after transfection. The Bend.3 cells transfected with PLKO.1-puro-PKCalpha-shRNA, PLKO.1-puro-PKCβ-shRNA, PLKO.1-puro-PKCζ-shRNA and empty PLKO.1-puro plasmids were called Bend.3/Sh-PKCa, Bend.3/Sh-PKCβ, Bend.3/Sh-PKCζ and Bend.3/vector-2 cells, respectively. All of them were used for experiments after selection by Puromycin (300 ug/ml, Sigma). The inhibition levels of RhoA activity and PKC expression were detected by pull-down assay and western blot, respectively.

2.2.3. 3TER assay

Endothelial permeability can be assayed by measuring transendothelial electrical resistance (TER). TER across the monolayers grown on the filter membranes was measured using the Millicell ERS Voltohmmeter (Millipore, JPN), and the values are shown as $\Omega\,cm^2$ based on culture inserts. The TER of cell-free inserts was subtracted from the TER of filters with cells.

2.2.4. Actin staining

Cells were stained with rhodamine-phalloidin (Invitrogen, U.S.) to examine the structure of filamentous (F)-actin. Washed cells were fixed with polyoxymethylene, washed again and permeabilized for 5 min with 0.1% Triton X-100. The cells were incubated with a 1% solution of BSA (30 min, RT), and stained with rhodamine-phalloidin (0.20 mol/L, 30 min, RT, in dark conditions). Stained F-actin was visualized using an OLYMPUS XB-51 fluorescence inverted microscope under 200-fold magnification.

2.2.5. Western immunoblotting

Western immunoblots were performed to measure the changed expression levels of tight junction proteins claudin-5 and ZO-1. Samples were diluted 1:4 with sample buffer and boiled for 3 min. Protein from each sample was loaded at 30 μg/well; ZO-1 and claudin-5 were run on 8% and 12% gels, respectively. After electrophoresis, protein was transferred onto a polyvinylidene difluoride membrane (PVDF). Membranes were blocked by 5% BSA for 2 h at room temperature and then incubated overnight at 4 °C [anti-claudin-5 (1:500) and ZO-1 (1:300)]. Secondary antibody was incubated for 1 h at room temperature. A chemiluminescence reagent, ECL western blotting detection reagent, was used to make the labeled protein bands visible.

2.2.6. Immunofluorescence

Cells were fixed with 100% methanol for 5 min at room temperature and then treated with PBS containing 0.5% Triton-X100. Subsequently, the cells were allowed to react with rabbit polyclonal antibody against claudin-5 (1:100) at 4 °C overnight. After washing with PBS, they were incubated with cy3-conjugated swine polyclonal antibody against rabbit immunoglobulins (DakoCytomation Denmark) for 2 h at room temperature. The stained cells were then washed with PBS and mounted in 60% glycerol-PBS for observation under a confocal microscope (FluoView FV1000; Olympus).

2.2.7. RhoA activity assay

RhoA activity was measured using a RhoA pull-down kit according to the manufacturer's protocols. Briefly, cell cultures were lysed in 500 μl of the supplied lysis buffer. Equal volumes of supernatants were incubated with RhoA-RBD affinity beads for 1 h at 4 °C, followed by two washes in lysis buffer and three washes in the supplied wash buffer. Bound proteins were eluted in 5 × 1% SDS sample buffer and examined by 12% SDS–PAGE and western blot with anti-RhoA antibody analysis. Aliquots of total lysate were also analyzed for the amount of RhoA present.

2.2.8. PKC activity assay

PKC activity was measured using PKC assay kits. Cell cultures were lysed in 500 μl of NP-40 lysis buffer. The cell lysates were incubated in PKC-a, PKC-β and PKC-ζ antibody (2 μg antibody/800 μg protein) with a rotor shaker, at 4 °C overnight. Then 20 μl of ProteinA/G agarose were added into the cell lysate, which was then centrifuged and washed with PBS 5 times. After final aspiration, 5 μL of KRREILSRRPSYR substrate, 5 μL of the ATP solution and 15 μl of PKC kinase buffer were added to initiate the kinase reaction at 30–35 °C for 60 min with constant shaking. The kinase reaction was stopped with 20 μL 2 × SDS-sample loading buffer and boiled for 2 min. Then PKC activity was examined by 12% SDS–PAGE and western blot with anti- KRREILSRRPpSYR antibody analysis.

2.3. Statistical analyses

All data is expressed as mean ± SD. The statistical significance was assessed by using one-way ANOVA analysis followed by Student-Neuman–Keuls post hoc tests. Differences were considered to be statistically significant at $P < 0.05$.

3. Result

3.1. PKC and Rho signaling involved in LPS-mediated changes in BMEC permeability

A number of intracellular signaling pathways have been implicated in regulating endothelial cell permeability. In this study, we tested whether any of them are involved in LPS-induced changes in the permeability of BMECs by TER assay. Bend.3 cells were pretreated for 1 h with either calphostin C (1 μM), wortmannin (100 nM), C3 transferase (1 μg/ml) or PP2 (3 μM), which are inhibitors of PKC, PI3Ks, Rho, and tyrosine kinase, respectively. then their TER was measured in the presence of 1 μg/ml LPS for 0–24 h. The results show that administration of LPS resulted in a time-dependent decrease in TER, which was significantly blocked by pretreating with PKC and RhoA inhibitors, but not with the other kinase inhibitors tested (Fig. 1A).

Then, we took advantage of enzyme and pull-down assays to investigate the role of LPS in activating PKC and RhoA signaling. We found that LPS exposure induced rapid and prolonged RhoA activation in a time-course manner. RhoA activity increased significantly at 5 min, with a maximum response occurring at 30 min (Fig. 1B). Significant increases in PKC-a activity as well as PKC-β and PKC-ζ activity were also found in Bend.3 cells exposed to LPS (Fig. 1C).

3.2. LPS-mediated BMEC hyperpermeability related to PKC (a, β, ζ) and RhoA-dependent TJ alteration

To determine whether disruption of TJ proteins is involved in LPS-induced and PKC or RhoA-dependent BMEC hyperpermeability, the effects of ShRNA-PKC (a, β, ζ) and N19RhoA plasmid on TJ proteins (ZO-1, Claudin-5) and the cytoskeleton (F-actin) were detected. The significant knockdown effect of ShRNA-PKC a, β and ζ was confirmed by western blot, and the inactivating effect of N19RhoA plasmid was detected by pull-down assay (data not show). As shown in Fig. 2A and B, both depleting PKC (a, β, ζ) and inactivating RhoA significantly abrogate LPS-mediated TJ protein degradation and suppress claudin-5 removal from membrane to cytoplasm. We also found that F-actin rearrangement induced by LPS could be prevented by inhibiting PKC (a, β, ζ) and RhoA signaling (Fig. 3).

3.3. Interactions between PKC and RhoA signaling in LPS-induced BMEC barrier dysfunction

The data presented above indicate that PKC-a, PKC-β, PKC-ζ and RhoA signals are all involved in LPS-induced TJ hyperpermeability. To further reconstruct the signaling pathways triggered by LPS in Bend.3 cells, potential interactions between RhoA and PKC were examined. Experiments were performed in which RhoA activation

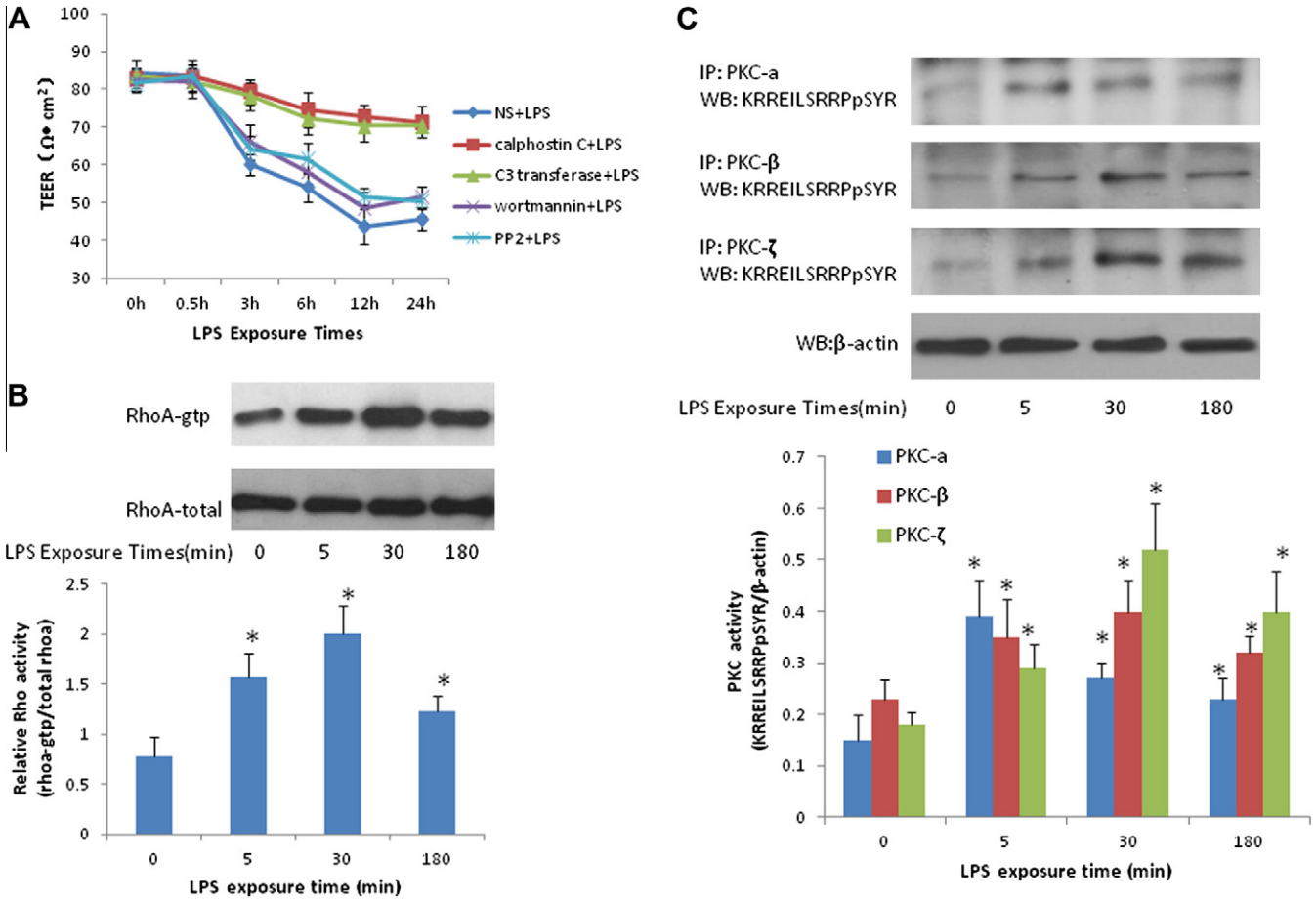

Fig. 1. Effects of PKC and Rho on LPS-mediated BMEC permeability changes. **A:** Bend.3 cells were pretreated with either calphostin C (1 μM), wortmannin (100 nM), C3 transferase (1 μg/ml) or PP2 (3 μM) for 1 h prior to LPS (10 μg/ml) exposure for 0–12 h. TER was determined. A significant increase in paracellular permeability was observed in cells exposed to LPS, and this increase was blocked by calphostin C and C3 transferase, but not other inhibitors tested. **B:** Pull-down assay was used to investigate the effect of LPS on activating RhoA signaling. LPS exposure induced rapid and prolonged RhoA activation in a time-course manner. **C:** Effect of LPS on activating PKC signaling was detected by enzyme assay. Significant increases in PKC-a activity as well as PKC-β and PKC-ζ activity were found in Bend.3 cells exposed to LPS compared with the control. Values are mean ±SEM (n = 3). *: $p < 0.05$ vs. Bend.3 cells in the absence of LPS in the same group.

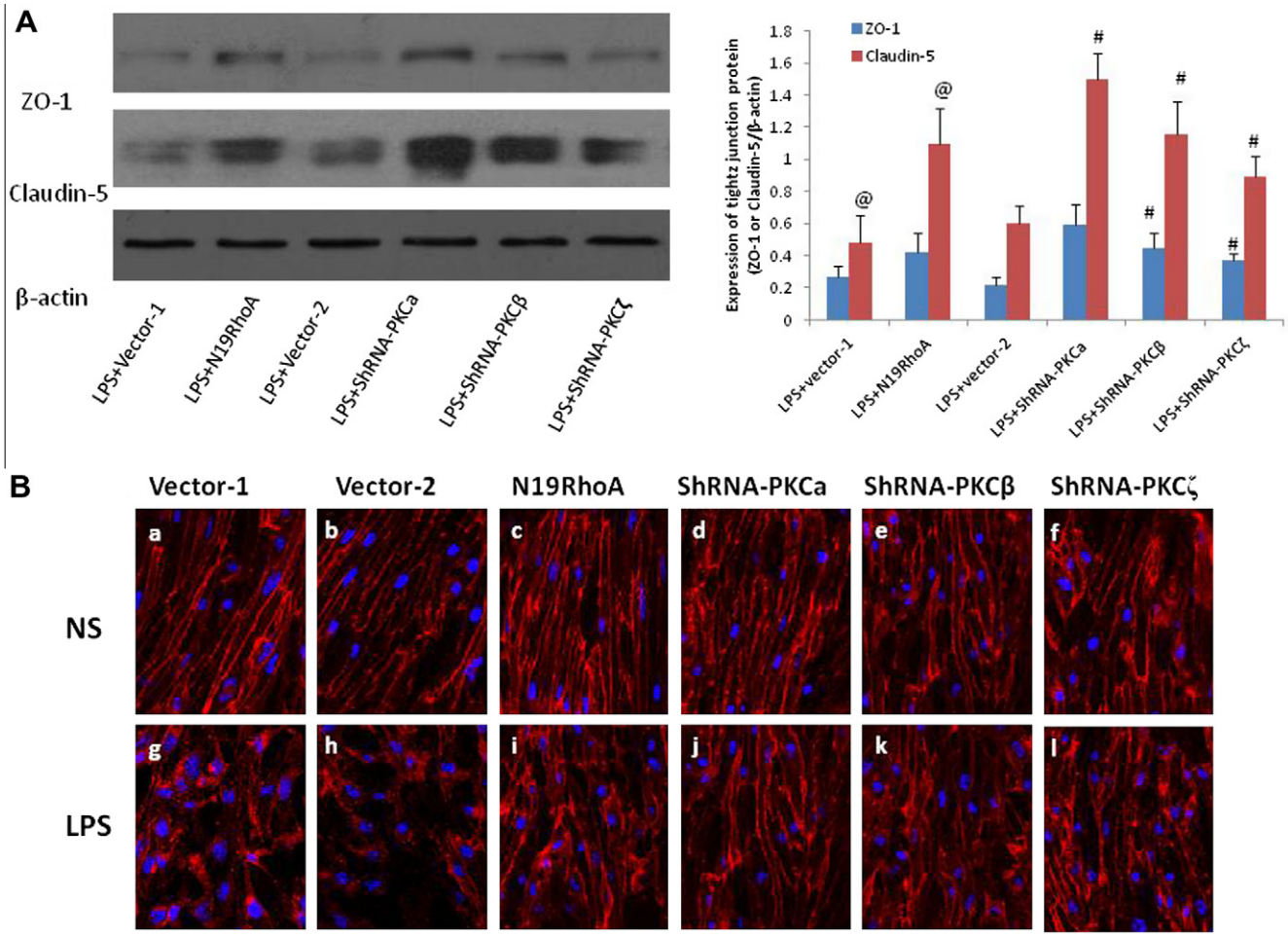

Fig. 2. The involvement of RhoA and PKC isoforms in LPS-induced decrease and redistribution of TJ protein. **A:** Bend.3 monolayers were first stably transfected either with the N19RhoA or ShRNA for PKC-a, PKC-β and PKC-ζ, or just with vector, and then the Bend.3 monolayers were exposed to LPS (for 12 h) and used to detect the expression levels of claudin-5 and ZO-1. Values are mean ± SEM (n = 3). @: $p < 0.05$ vs. Bend.3/vector-1 cells. #: $p < 0.05$ vs. Bend.3/vector-2 cells. Reducing PKC isoforms and RhoA activity reduced LPS-induced decrease of TJ protein expression. **B:** Stably transfected Bend.3 monolayers were exposed to LPS (for 0 h or 12 h) and used to detect the distribution of claudin-5. Reducing PKC isoforms and RhoA activity reduced LPS-induced redistribution of the claudin-5 protein.

was inhibited (by N19RhoA plasmid or C3 transferase) and the activity of PKC-a, β and ζ were observed by enzyme assay. We found that inhibition of RhoA significantly diminished PKC-ζ activation in the presence of LPS, but PKC-a and PKC-β were unaffected (Fig. 4A). Then opposite experiments were performed where ShRNA was used to deplete PKC, and RhoA activity was observed. The results showed that, depleting PKC-a prevented the activation of RhoA, whereas depleting PKC-ζ and PKC-β did not interrupt RhoA activation (Fig. 4B).

To further detect the relationship between PKC-a and RhoA does really exist in BMEC-TJ regulating process, Bend.3/N19RhoA cells and Bend.3/vector-1 cells were pretreated with Gö6976 (1 μM, a specific inhibitor of PKC-a) for 1 h, and then their TER was detected. The results showed that blocking PKC-a alone had an effect similar to that of blocking both PKC-a and Rho activity, which indicates PKC-a may act upstream of RhoA in regulating the brain endothelial barrier (Fig. 4C). Similarly, to confirm whether PKC-ζ acts as downstream target of RhoA in the process, Bend.3/Sh-PKCζ and Bend.3/vector-2 cells were pretreated with C3 transferase for 1 h, then TER detection was performed as mentioned before. As shown from Fig. 4D, comparing results obtained from blocking both of these signal molecules with data from inhibiting RhoA activity alone indicates promotion of PKCζ activity partly related to RhoA activation in the process of LPS-mediated Bend.3 cell TJ dysfunction.

4. Discussion

Previous studies have shown that LPS can increase BBB permeability in vivo and brain endothelial barrier permeability in vitro, which is the prominent pathological feature of the last meningeal inflammation step [12]. Our study further examined the signaling pathways activated by LPS to enhance brain endothelial barrier permeability. The results demonstrate the following. (1) PKC and RhoA, but not PI3 Ks and tyrosine kinase, are involved in LPS-mediated BMEC permeability promotion. (2) PKC isoforms (a, β, ζ) and RhoA are activated by LPS and alter BMEC tight junctions. (3) With respect to LPS-induced BMEC hyperpermeabiltiy, the PKC-a and PKC-ζ signals act as upstream and downstream targets for the RhoA signal, respectively, whereas PKC-β does not interact with RhoA.

TJ structures are frequently modified by a network of signaling pathways including PKC, PI3Ks, Rho, and tyrosine kinase, and leading to an increase in permeability. For example, applying PI3K inhibitor LY294002 and wortmannin can reduce VEGF or hypoxia -mediated ZO-1 and occludin redistribution and promote TER [13,14]. Our previous studies also found that RhoA and PKC are important regulators for TJs in inflammation [11,15]. However, signaling networks are complicated and may vary according to the different stimulus and cell types. Take the Rho/ROCK pathway, for instance. It has been recognized as participating in the

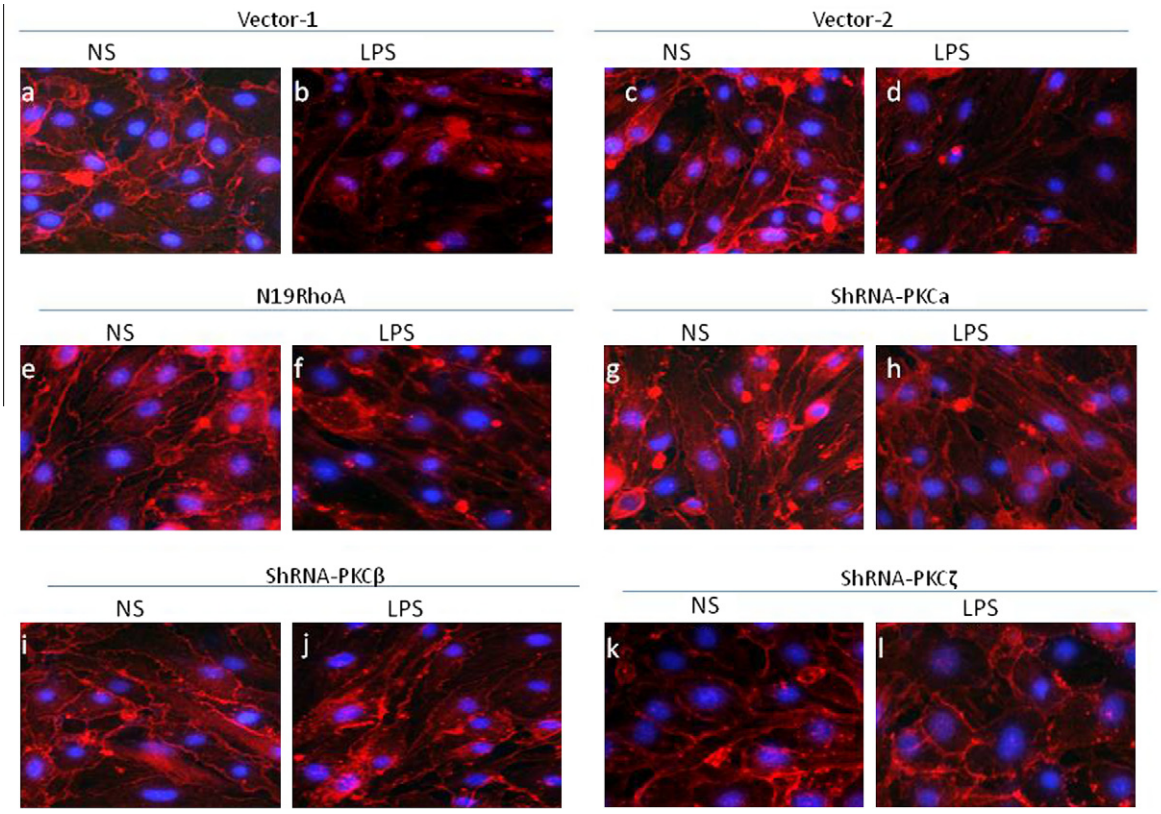

Fig. 3. The involvement of RhoA and PKC isoforms in LPS-induced F-actin rearrangement. Stably transfected Bend.3 monolayers were exposed to LPS (for 0 h or 12 h) and used to detect F-actin dynamics. Reducing PKC isoforms and RhoA activity reduced LPS-induced reorganization of F-actin.

regulation of cytoskeletal dynamics and TJ assembly, and thus inference, in the control of endothelial permeability. However, the involvement of Rho/ROCK signaling in TJ permeability has recently been challenged. It reported that inhibition of the RhoA/ROCK pathway had little effect on improving an LPS-induced increase in skeletal muscle permeability [16], and inhibitor of ROCK does not counteract histamine-induced microvascular leakage in the airway [17].

In our study, we found that depressors of PKC and Rho prevented LPS-induced BMEC hyperpermeability, but inhibitors of PI3Ks and tyrosine kinase had less of a promoting effect on TER. PKC and RhoA could be activated by LPS treatment. These data show that PKC and RhoA signals, but not PI3Ks and tyrosine kinase signals, are involved in LPS-mediated BMEC tight junction's permeability promotion. In addition, we utilized ShRNA and dominant negative plasmid approaches to investigate whether PKC and RhoA signals regulate BMEC permeability by modifying the TJ complex. The results show that both depleting PKC and inhibiting RhoA improve LPS-induced TJ disruption and F-actin reorganization, indicating that PKC and RhoA transfer LPS disruptive signals to the TJs, leading to BMEC dysfunction.

Another key question addressed by our study is which PKC isoforms transfer LPS disruptive effects to the TJs? The PKC family consists of at least 11 isoforms which differ in structure, substrate requirement, and cell localization and are involved in the regulation of many different cell functions [18,19]. Different PKC isoforms have been shown to induce either assembly or disassembly of tight junctions depending on the cell type and conditions of activation [20–22]. A number of well-documented studies have indicated that cPKC and aPKC constitute part of the tight junction complex [23,24]. Among the cPKC and aPKC subfamilies, PKC-a, PKC-β and PKCa-ζ are thought to be mainly expressed in capillary endothelium and CNS [25,26], so we chose these three PKC isoforms as our target. In our experiment, LPS induced activation of PKC-a, PKC-β and PKCa-ζ, and through them caused morphological and biochemical alterations in the TJs of BMECs. The inhibition study clearly indicates the obligatory role of PKC-a, PKC-β and PKCa-ζ isoforms in regulating the TJ complex by LPS.

Based on previous data and the experiments described above, it appears that both RhoA and PKC signaling molecules are involved in the process. They have the same end points, disruption of TJ proteins and rearrangement of F-actin, leading to brain endothelial barrier dysfunction. The final question is, how about the regulatory relationship between them? It is a controversial topic. For instance, in vitro protein–protein interaction studies have shown that RhoA can regulate PKC-a activation [27,28]. However, it also has been reported that conventional PKC phosphorylates Rho-GDP dissociation inhibitor (GDI) on serine 34, resulting in an increase of RhoA activity [29]. Our outcomes show that deletion of PKC-a suppressed the LPS-induced promotion of RhoA activity, whereas deletion of PKC-β and PKC-ζ could not prevent RhoA activation. Meanwhile, consistent inhibition of RhoA had no effect on PKC-a and PKC-β activity, but could inhibit LPS-induced PKC-ζ activation. Furthermore, blocking two signal molecules such as PKC-a and RhoA or RhoA and PKC-ζ led to a TER result similar to inhibiting PKC-a or RhoA activity alone. These data suggest that PKC-a and PKC-ζ, but not PKC-β, interact with RhoA to increase brain endothelial barrier permeability, and that PKC-a and PKC-ζ are the upstream and downstream regulators for RhoA, respectively.

Collectively, our data indicates LPS affects the brain endothelial TJ barrier in a manner which is dependent on PKC (a, β, ζ) and RhoA, but independent of the PI3K and tyrosine kinase pathways. PKC-a and PKC-ζ, respectively, act as the upstream and downstream regulators for RhoA in the process. Further studies of the

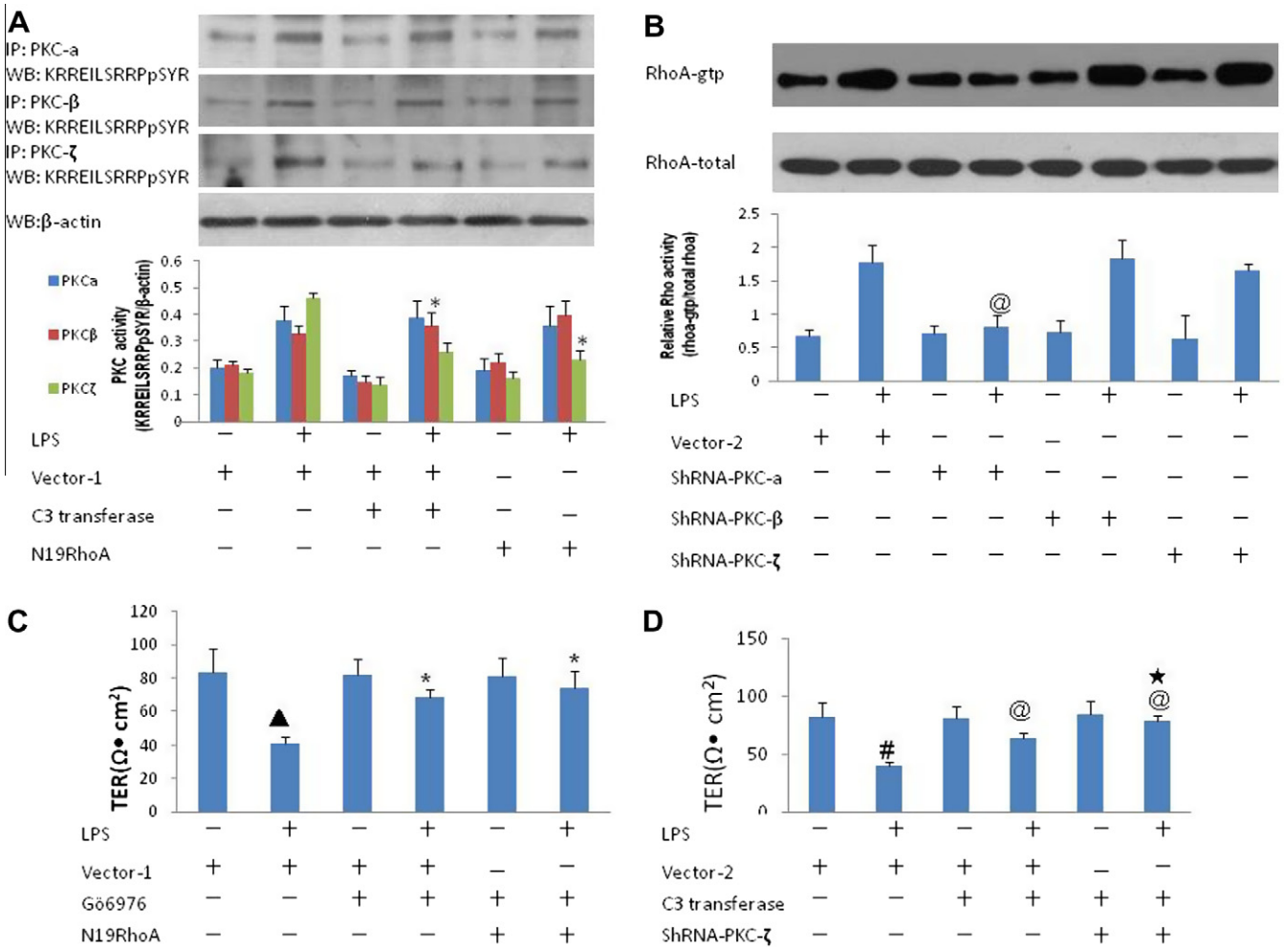

Fig. 4. Relationship between PKC and RhoA in regulating LPS induced hyperpermeability of brain endothelial cells, **A:** Effect of inhibition of Rho activity on PKC activation .Stably transfected Bend.3/vector-1 cells were pretreated with C3 transferase for 1 h. Then Bend.3/vector-1 + C3 transferase cells and stably transfected Bend.3/N19RhoA cells were exposed to LPS for 0 min or 30 min and the activities of PKC-a, PKC-β and PKC-ζ were detected by enzyme assay. Consistent inhibition of RhoA had no effect on PKC-a and PKC-β activity, but inhibited LPS-induced PKC-ζ activation. Values are mean ± SEM (n = 3). ∗: $p < 0.05$ vs. Bend.3/vector-1 cells treated with LPS. **B**: Effect of depletion of PKC isoforms on RhoA activation in brain endothelial cells. Stably transfected Bend.3/Sh-PKCa, Bend.3/Sh-PKCβ, Bend.3/Sh-PKCζ and Bend.3/vector-2 cells were exposed to LPS for 0 min or 30 min and the activities of RhoA were detected by pull-down assay. Deletion of PKC-a suppressed the LPS-induced promotion of RhoA activity, whereas deletion of PKC-β and PKC-ζ could not prevent RhoA activation. Values are mean ± SEM (n = 3). @: $p < 0.05$ vs. Bend.3/vector-2 cells treated with LPS. **C:** The "PKC-a/RhoA" pathways play critical roles in LPS-induced increase in brain endothelial permeability. Bend.3/N19RhoA cells and Bend.3/vector-1 cells were pretreated with Gö6976 (1 μM) for 1 h, and their TER was detected after LPS treatment (0 h or 12 h). Blocking PKC-a alone had an effect on TER similar to that of blocking both PKC-a and Rho activity. Values are mean ±SEM (n = 3). ▲: $p < 0.05$ vs. Bend.3/vector-1 cells in the absence of LPS. ∗: $p < 0.05$ vs. Bend.3/vector-1 cells treated with LPS. **D:** The "RhoA/PKC-ζ" pathways play critical roles in LPS-induced increase in brain endothelial permeability. Bend.3/Sh-PKCζ and Bend.3/vector-2 cells were pretreated with C3 transferase (1 μM) for 1 h, then TER detection was performed as mentioned in Fig. 4C. Compared to blocking PKC-ζ activity alone, inhibiting both PKC-ζ and Rho activity had stronger repair effect on TER, which indicated promotion of PKCζ activity partly related to RhoA activation in the process of LPS-mediated Bend.3 cell TJ dysfunction.Values are mean ±SEM (n = 3). #: $p < 0.05$ vs. Bend.3/vector-2 cells in the absence of LPS. @: $p < 0.05$ vs. Bend.3/vector-2 cells treated with LPS, ★: $p < 0.05$ vs. Bend.3/vector-2 cells treated with LPS and pretreated with C3 transferase.

specific signaling events that occur in this process may contribute to finding new drug targets for bacterial meningitis.

Acknowledgments

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