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BRAIN RESEARCH

Research Report

Role of p115RhoGEF in lipopolysaccharide-induced mouse brain microvascular endothelial barrier dysfunction

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ABSTRACT

Background: In endothelial cells, exposure to lipopolysaccharide (LPS) results in barrier dysfunction through a complex signaling mechanism. The RhoA/Rho-kinase pathway plays a significant role in endothelial cell permeability. p115RhoGEF, a specific guanine nucleotide exchange factors (GEFs) activates RhoA, triggering RhoA-dependent cytoskeletal remodeling. However, little is known about the role of p115RhoGEF in LPS-induced brain endothelial barrier breakdown. We hypothesized that suppression of p115RhoGEF may inhibit activation of RhoA and prevent LPS-induced brain microvascular endothelial cell hyperpermeability. *Methods:* The cultured monolayer of bEnd.3 cells, an immortalized mouse brain endothelial cell line, was used in this study. bEnd.3 cells were pretreated with specific siRNA to knockdown p115RhoGEF or C3 transferase to inhibit RhoA activity, and then incubated with LPS (5 μg/ml). The degree of RhoA activation was determined by a Rhotekin-based pull-down assay, and expression of p115RhoGEF, zonula occludens-1 (ZO-1), occludin and claudin-5 proteins were detected by Western blot analysis. The barrier function was measured by transendothelial electrical resistance (TEER). F-actin cytoskeleton was visualized by Rhodamine-phalloidin staining. *Results:* The expression level of p115RhoGEF protein was significantly increased in LPS-treated bEnd.3 cells. The activity of RhoA was enhanced after LPS stimulation and pretreatment with p115RhoGEF siRNA or exoenzyme C3 transferase reduced RhoA activation significantly as shown by the pull-down assay. Furthermore, depletion of p115RhoGEF partially prevented the LPS-induced decrease in TEER, stress fiber formation and tight junction proteins degradation. *Conclusions:* These results suggest that p115RhoGEF is important for LPS signaling to RhoA and LPS-induced endothelial barrier dysfunction, providing new insight into the function of RhoGEFs in inflammation.

1. Introduction

The brain microvascular endothelial cells (BMEC) play a vital role in maintaining the stability of the central nervous system. The restrictive characteristic of BMEC is conferred by tight junction (TJ) proteins, which mainly consist of occludin, claudins, and zonula occludens (Wolburg and Lippoldt, 2002). During Gram-negative sepsis, lipopolysaccharide (LPS) is responsible for endothelial cell changes including actin rearrangement, tight junction proteins redistribution, and cell detachment from the extracellular matrix (Goldblum et al., 1993; Harlan et al., 1983).

The small guanosine triphosphate (GTP)-binding protein RhoA is a critical mediator of cytoskeletal organization, stress

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fiber formation, and myosin light chain (MLC) phosphorylation in a variety of cell types (Wojciak-Stothard and Ridley, 2002; Clements et al., 2005). Several agonists appear to induce endothelial cell hyperpermeability through the RhoA/Rho kinase pathway. Inhibition of RhoA activity by C3 transferase prevented thrombin-induced loss of TJ proteins and formation of stress fibers in human umbilical vein endothelial cells (HUVEC) (Wojciak-Stothard et al., 2001). In addition, pretreatment with Y-27632, a specific inhibitor of Rho kinase, attenuated cytoskeletal rearrangement of pulmonary artery endothelial cells and pulmonary edema in mouse after LPS challenge (Tasaka et al., 2005).

The activity of RhoA is controlled by RhoGEFs which promote the release of guanosine diphosphate (GDP) in exchange for guanosine triphosphate (GTP). Among these many RhoGEFs, the discovery of p115RhoGEF provided a first-link between α subunit of G-protein 12/13 (Gα12/13) with RhoA (Hart et al., 1998). The Gα13/p115RhoGEF/RhoA pathway was proven to be directly involved in thrombin-induced microtubule network disassembly in pulmonary artery endothelial cells (Birukova et al., 2004) and stress fiber formation in HUVEC (Holinstat et al., 2003). Furthermore, it is indicated that Rho activation depends on the convergence of protein kinase Cα (PKCα) and Gα12/13 signaling pathways at p115RhoGEF (Holinstat et al., 2003). Although studies in peripheral endothelial cell lines have shown that RhoA is involved in LPS-induced barrier injury and p115RhoGEF is a critical regulator of RhoA, the role of this signaling pathway has not been studied in brain.

Using an *in vitro* model of mouse BMEC, we tested the hypothesis that LPS contributes to barrier dysfunction via the activation of p115RhoGEF/RhoA-dependent signaling. In the current study, inhibition of p115RhoGEF with siRNA significantly reduced RhoA activation and attenuated endothelial barrier injury evoked by LPS.

2. Results

2.1. *p115RhoGEF expression is upregulated in LPS-treated bEnd.3 cells*

bEnd.3 cells were incubated with LPS (5 μg/ml) for 1, 2, 3, 6, and 12 h. The upregulation of p115RhoGEF was detected as early as 1 h after start of LPS treatment and expression continuously increased up to 6 h. The expression level of p115RhoGEF fell back to its normal range by the time point 12 h (Fig. 1).

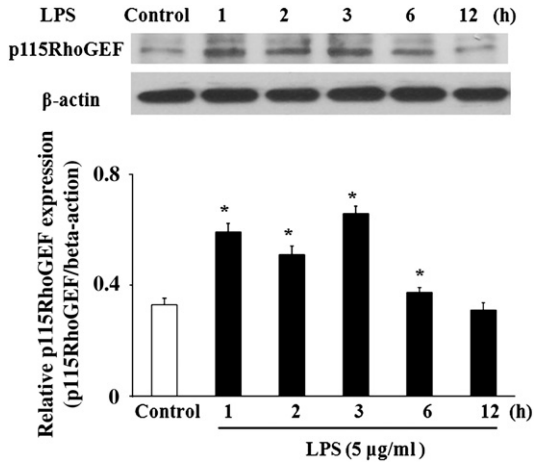

Fig. 1 – p115RhoGEF expression is upregulated after LPS treatment. At 1, 2, 3, 6, and 12 h after incubation with LPS (5 μg/ml), bEnd.3 cells were lysed and total cell proteins were assayed by immunoblotting. Representative blots for p115RhoGEF and β-actin are shown. The expression levels of p115RhoGEF are summarized in the lower panel. Values are means ± SEM ($n=3$). *$P<0.05$ vs. control group.

2.2. *LPS-induced RhoA activation is reduced in p115RhoGEF siRNA and C3 transferase-pretreated bEnd.3 cells*

bEnd.3 cells were divided into 3 groups: nsRNA, siRNA, and C3. The depletion of p115RhoGEF protein by siRNA was confirmed by Western blot analysis (Fig. 2A). RhoA activity was increased 115% for 5 min following treatment with LPS (Fig. 2B). p115RhoGEF siRNA and C3 transferase, respectively, reduced RhoA activation by 70% and 91% after 5 min LPS treatment ($P<0.05$ vs. nsRNA plus LPS group, Fig. 2B). RhoA activity displayed a remarkable reduction in C3 group as compared with siRNA group ($P<0.05$ vs. C3 plus LPS group, Fig. 2B).

2.3. *LPS-induced disruption of barrier function is attenuated in p115RhoGEF siRNA and C3 transferase-pretreated bEnd.3 cells*

Barrier function of endothelial cells in response to LPS (5 μg/ml) was assessed by measuring the TEER on the endothelial monolayer at 0.5, 1, 3, 6, 12, and 24 h. Pretreatment of cells with nsRNA alone had no significant effect on TEER. LPS resulted in a time-dependent decrease of TEER in the nsRNA group. Compared with the control nsRNA group (81.5 ± 2.47 Ω cm^2), LPS perturbed the integrity of bEnd.3 cell monolayer leading to a significant decrease in TEER at the end of the 3-h incubation period (55.3 ± 2.08 Ω cm^2) ($P<0.05$). After stimulated with LPS for 24 h, the TEER of bEnd.3 cells decreased to 48.3% of baseline (39.2 ± 1.48 Ω cm^2). Pretreatment with p115RhoGEF siRNA and C3 transferase significantly abrogated the effect of LPS. TEER of siRNA group and C3 group, respectively, were 65.4 ± 2.01 Ω cm^2 and 72.3 ± 1.63 Ω cm^2 compared to 55.3 ± 2.08 Ω cm^2 for nsRNA group at 3 h.

2.4. *LPS-induced changes in F-actin are attenuated in p115RhoGEF-depleted bEnd.3 cells*

The changes of F-actin were investigated by immunofluorescence, which were shown in Fig. 4. Before LPS treatment, bEnd.3 cells did not display prominent stress fibers (Fig. 4A and B). Three-hour LPS exposure caused stress fiber formation and paracellular gaps between cell monolayer (Fig. 4C), whereas F-actin structure remained unaffected in cells transfected with p115RhoGEF siRNA (Fig. 4D).

2.5. *LPS-induced degradation of TJ proteins is reduced in p115RhoGEF siRNA and C3 transferase-pretreated bEnd.3 cells*

The cellular protein levels of ZO-1, occludin, and claudin-5 decreased in a time-dependent manner under treatment with