Biochemical and Biophysical Research Communications 417 (2012) 925–930

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

cPLA$_2\alpha$-mediated actin rearrangements downstream of the Akt signaling is required for *Cronobacter sakazakii* invasion into brain endothelial cells

Dong-Xin Liu, Wei-Dong Zhao, Wen-Gang Fang, Yu-Hua Chen *

Department of Developmental Biology, Key Laboratory of Cell Biology, Ministry of Public Health, and Key Laboratory of Medical Cell Biology, Ministry of Education, China Medical University, 92 Bei Er Road, Heping District, Shenyang 110001, China

ARTICLE INFO

Article history:
Received 11 November 2011
Available online 25 November 2011

Keywords:
cPLA$_2\alpha$
Cronobacter sakazakii
Brain endothelial cells
Meningitis
Akt
Actin rearrangements

ABSTRACT

Cronobacter sakazakii (*C. sakazakii*) is an opportunistic pathogen that causes sepsis and meningitis in neonate. The molecular mechanism involved in the pathogenesis of *C. sakazakii* meningitis remains unclear. In this study, we found that *C. sakazakii* invasion was significantly decreased in human brain microvascular endothelial cells (HBMEC) treated with cytosolic phospholipases A$_2\alpha$ (cPLA$_2\alpha$) inhibitor. Increased phosphorylation of cPLA$_2\alpha$ was observed in HBMEC infected with *C. sakazakii*, which was prevented by treatment with cPLA$_2\alpha$ inhibitor. cPLA$_2\alpha$ knockdown in HBMEC significantly attenuated *C. sakazakii* invasion into HBMEC. Immunofluorescence demonstrated that the rearrangements of actin filaments in HBMEC induced by *C. sakazakii* were effectively blocked by either treatment with cPLA$_2\alpha$ inhibitor or transfection with cPLA$_2\alpha$ siRNA. Interestingly, we found that *C. sakazakii* infection promoted the aggregation of phosphorylated cPLA$_2\alpha$, which was associated with depolymerized actin filaments in HBMEC. Furthermore, our data revealed that cPLA$_2\alpha$ acts downstream of Akt signaling pathway in HBMEC stimulated with *C. sakazakii*. Taken together, our results illustrated that cPLA$_2\alpha$-mediated actin filament rearrangements downstream of Akt activation is required for *C. sakazakii* invasion into brain endothelial cells.

1. Introduction

Cronobacter sakazakii (*C. sakazakii*), formerly known as *Enterobacter sakazakii*, is an opportunistic pathogen that has been isolated from powdered infant formulas. *C. sakazakii* infection causes necrotizing enterocolitis, septicemia and meningitis, particularly in the premature and low-birth-weight infants [1,2]. It has been reported that most neonates who survive from *C. sakazakii*-induced meningitis suffer severe neurologic sequelae including hydrocephalus, quadriplegia, and retardation of development [3]. The mechanism of the pathogenesis of *C. sakazakii*-induced meningitis remains largely unclear.

It is known that bacterial penetration through the blood–brain barrier (BBB) into the brain is a key step during the pathogenesis of bacterial meningitis. Brain microvascular endothelial cells, which is the major components of BBB, are commonly used to study the molecular mechanisms of microbial–host interactions involved in bacterial meningitis [4]. We previously found that host cell actin filaments rearrangements are required for the invasion of brain endothelial cells by *C. sakazakii* [5]. However, the detailed mechanisms underlying *C. sakazakii* invasion of brain endothelial cells remain to be determined.

Phospholipase A$_2$ (PLA$_2$) designates a class of enzymes that hydrolyzes the sn-2 ester of glycerophospholipids to produce a fatty acid and a lysophospholipid. It is known that PLA$_2$ plays a significant role in many pathophysiological processes such as barrier function, eicosanoid production, and inflammation [6]. Previous studies showed that host PLA$_2$ is activated in macrophage infected with *Mycobacterium tuberculosis* and *Listeria monocytogenes*, epithelial cells infected with *Shigella flexneri*, and endothelial cells infected with *Escherichia coli* [7–10]. However, whether PLA$_2$ is involved in *C. sakazakii* invasion of host cells remains unknown. In this study, we identified the activation of host cPLA$_2\alpha$ in brain endothelial cells infected with *C. sakazakii*. Our data demonstrated that cPLA$_2\alpha$, acts downstream of Akt, is necessary for actin filaments rearrangements during *C. sakazakii* invasion of brain endothelial cells.

2. Materials and methods

2.1. Bacteria and culture conditions

The *C. sakazakii* type strain (ATCC 29544) was obtained from American Type Culture Collection (Manassas, VA). *C. sakazakii* was grown in brain–heart infusion broth (BD Biosciences) at 37 °C overnight without shaking.

* Corresponding author. Fax: +86 24 23260246.
E-mail address: yhchen@mail.cmu.edu.cn (Y.-H. Chen).

0006-291X/$ - see front matter Crown Copyright © 2011 Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.11.079

2.2. Cell culture

Human brain microvascular endothelia cells (HBMEC) were a generous gift from Dr. K.S. Kim (Johns Hopkins University School of Medicine, USA). They were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Hyclone), 10% Nu-serum (BD Biosciences), 2 mM glutamine, 1 mM sodium pyruvate, 1× non-essential amino acid and 1× MEM vitamin. The cells were incubated at 37 °C in a 5% CO_2, 95% air humidified atmosphere. Stable HBMEC cell line transfected with dominant-negative phosphatidylinositol 3-kinase (PI3K) (p110γΔ948–981) was constructed as described previously [11].

2.3. Bacterial invasion and adhesion assays

C. sakazakii invasion of HBMEC was performed as described previously [12]. In brief, bacteria (10^7 CFU/well) were added to confluent monolayers of HBMEC in 24-well plates at a multiplicity of infection of 100. The monolayers were incubated at 37 °C for 1.5 h to allow invasion occurring. The intracellular bacteria was determined after the extracellular bacteria were killed by incubation with experimental medium (RPMI1640, 5% fetal bovine serum and 1 mM sodium pyruvate) containing gentamicin (100 μg/ml) for 1 h at 37 °C. Then the monolayers were washed and lysed with 0.5% Triton X-100. The released intracellular bacteria were enumerated by plating on Druggan–Forsythe–Iversen (DFI) agar. For inhibitor studies, HBMEC were incubated with *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA, Sigma–Aldrich), AACOCF3 (Calbiochem), bromoenol lactone (BEL), CAY10590 (Cayman Chemical) and Akt1/2 kinase inhibitor (Bio Basic Inc.) for 30 min, respectively, before the addition of bacteria. HBMEC treated with vehicle (DMSO) were used as a control. Bacterial adhesion assay to determine the number of total cell-associated bacteria was done as described above except that the gentamicin step was omitted. Data are presented as the percentage of control.

2.4. Immunofluorescence

HBMEC grown on coverslips were incubated with *C. sakazakii* at 37 °C for 30 min. The cells were washed with PBS and fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 0.1% Triton X-100 and then blocked with 5% BSA. Then, the cells were incubated with TRITC-labeled phalloidin (Sigma–Aldrich) to stain the actin filaments. The coverslips were mounted and analyzed using immunofluorescence microscope (Olympus BX51).

For $cPLA_2\alpha$ analysis, the cells were prepared in the similar way, but were stained with antibody against phosphorylated $cPLA_2\alpha$ on Ser505 (p-$cPLA_2\alpha$) (Cell Signal Technology) followed by incubation with a secondary antibody conjugated to Alexa 488 (Invitrogen). The nuclei were stained with DAPI. The coverslips were analyzed using confocal laser scanning microscope (Olymupus FluoView FV1000). A line profile analysis for the interested areas in fluorescence image was carried out with Axiovision release 4.5 software (Carl Zeiss). When indicated, HBMEC were incubated with AACOCF3 (40 μM) for 30 min prior to *C. sakazakii* infection.

2.5. RNA interference

Three siRNAs sequences were designed based on the *$cPLA_2\alpha$* mRNA (NM_024420) sequence. siRNA-1 targeting to human $cPLA_2\alpha$ corresponded to nucleotides 945–963 (5′-GGCCAGAGGAGATTAATGA-3′), siRNA-2 corresponded to nucleotides 1687–1705 (5′-GGGCTTGAATCTCAATACA-3′), siRNA-3 corresponded to nucleotides 2059–2077 (5′-GGAGTGCTATGTCTTTAAA-3′), Non-silencing-siRNA (5′-TTCTCCGAACGTGTCACGT-3′) was used as control. The siRNAs were obtained from Genepharma Corp (Shanghai, China) and transiently transfected into HBMEC using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The knockdown of $cPLA_2\alpha$ in the transfected HBMEC was analyzed by Western blot.

2.6. Western blot

Cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) containing protease inhibitor cocktail (Roche). The samples were subjected to SDS–PAGE and then transferred to PVDF membrane (Millipore). The PVDF membrane was blocked with 5% non-fat milk and probed with the indicated antibody at 4 °C overnight. Then, the blots were incubated with an HRP-conjugated secondary antibody (Santa Cruz Biotech.) for 1 h at room temperature. Immunoreactive bands were visualized by Super Signal West Pico Chemilumine-scent Substrate (Pierce) using LAS3000mini (Fuji Film). For quantitative analysis, the mean density of each band was measured by Multi Gauge V3.1 software, and the band density of activated form of the protein was divided by the density of the corresponding total protein band to obtain the normalized band density. Antibodies against $cPLA_2\alpha$ and $cPLA_2\alpha$ with phosphorylated Ser505 (p-$cPLA_2\alpha$) were obtained from Cell Signal Technology. Anti-Akt antibody was from Millipore. Antibody against Akt with phosphorylated Ser473 (p-Akt) was from Santa Cruz Biotech.

2.7. Statistical analysis

Statistical significance between two groups was analyzed by Student's *t* test. One-way ANOVA was used to compare multiple groups.

3. Results

3.1. *$cPLA_2\alpha$* is associated with *C. sakazakii* invasion of HBMEC

To identify whether PLA_2 is involved in *C. sakazakii* invasion of HBMEC, bacterial invasion were performed in the presence of ACA [13], a non-selective PLA_2 inhibitor. As shown in Fig. 1A, the invasion of *C. sakazakii* into HBMEC was significantly inhibited by ACA treatment. ACA inhibitor could block *C. sakazakii* invasion of HBMEC in a dose-dependent manner with a 49% inhibition at 80 μM. In contrast, the adhesion of *C. sakazakii* to HBMEC was not affected by ACA treatment. These results implicated a role for host PLA_2 in *C. sakazakii* invasion of HBMEC. It was known that PLA_2 has been classified into three major categories in mammalian cells, including secretory PLA_2 ($sPLA_2$), intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$) and cytosolic PLA_2. To determine which type of PLA_2 is associated with *C. sakazakii* invasion of HBMEC, CAY10590 [14,15], BEL [15] and AACOCF3 [16,17] were used to inhibit the activity of $sPLA_2$, $iPLA_2$ and $cPLA_2\alpha$ in HBMEC, respectively, followed by bacterial invasion and adhesion assay. The results showed that inhibition of $sPLA_2$ and $iPLA_2$ had no effect on the adhesion to and invasion of HBMEC by *C. sakazakii* (Fig. 1B and C). Interestingly, AACOCF3, which has been identified as a $cPLA_2\alpha$ specific inhibitor [16,17], effectively prevented *C. sakazakii* invasion of HBMEC in a dose-dependent manner with a 62% inhibition at 40 μM (Fig. 1D). In contrast, inhibition of $cPLA_2\alpha$ had no effect on *C. sakazakii* adhesion to HBMEC (Fig. 1D). These results suggested that host $cPLA_2\alpha$ plays a role in *C. sakazakii* invasion of HBMEC.